40. A method according to claim 39, wherein said first half-site is obtained from an ecdysone response element and said second half-site is obtained from a glucocorticoid response element.

41. A method according to claim 40, wherein said first half-site is AGTGCA and said second half-site is TGTTCT.

42. A method according to claim 13, wherein said ecdysone response element has the sequence AGTGCA-N-TGTTCT.

43. A formulation according to claim 27, wherein said composition is effective to activate at least one member of the steroid/thyroid hormone receptor superfamily.

44. A formulation according to claim 43, wherein said member is an ecdysone receptor.

45. A formulation according to claim 44, wherein said formulation acts to modulate gene transcription for a gene maintained under the control of an ecdysone response element.

46. A formulation according to claim 27 wherein said ecdysteroid is 20-hydroxyecdysone.

REMARKS

The present invention is based on the discovery that optimized promoters containing a novel modified ecdysone response element in conjunction with an invention modified ecdysone receptor (preferably having an altered DNA binding specificity) provide an extremely powerful and specifically inducible mammalian expression system.

Accordingly, the present invention provides method(s) for modulating expression of exogenous gene(s) in mammalian subject(s) containing a defined DNA construct. DNA constructs contemplated herein comprise an exogenous gene under the control of an ecdysone response element plus a modified ecdysone receptor which, in the presence of an appropriate

ligand, binds to the ecdysone response element, and optionally a further receptor which, in the presence of the modified ecdysone receptor, can act as a silent partner. The invention method comprises administering to a suitable mammalian subject an effective amount of a ligand for the modified ecdysone receptor that is not normally present in the cells of the subject. In the presence of ligand for the modified ecdysone receptor, (and optionally in the presence of a receptor that can act as a silent partner), the ligand binds to the receptor, promoting its interaction with invention modified ecdysone response element, thereby modulating expression of the exogenous gene. The present invention also provides formulations containing at least one ecdysteroid and a pharmaceutically acceptable carrier.

By this communication, claims 25, 31 and 34 have been amended and new claims 35-46 have been added in order to define Applicants' invention with greater particularity. No new matter is presented by the claim amendments as the new claim language is fully supported by Applicants' specification and original claims.

Thus, upon entry of the amendment, claims 1-46 will be pending, and are presented for the Examiner's convenience as Exhibit A.

Applicants respectfully disagree with the Examiner's assertion that the present invention "lie[s] in the realm of gene therapy" (Office Action in the prior application, dated July 2, 1999). Contrary to the Examiner's assertion, all that is required by claim 1 (and claims 2-21 and 35-42, dependent thereon) is administering to a suitable subject an effective amount of a ligand for invention modified ecdysone receptor(s), wherein the ligand is not normally present in the cells of the subject, and wherein the ligand is not toxic to the subject, to modulate the expression of the exogenous gene in the subject. To the extent that a mammal must be treated to become a subject suitable for practice of the invention methods, that is a precondition that must be satisfied before one could carry out Applicants' invention methods. One cannot fall within the scope of the claims unless the precondition has been met.

Accordingly, Applicants respectfully submit that the present claims are not in the realm of gene therapy because they do not require several of the process steps that gene therapy would

necessarily require. For example, claim1 (and claims dependent thereon) do not require stable insertion into a mammalian subject of a DNA construct comprising an exogenous gene under the control of an ecdysone response element. Claim 1 does not additionally require insertion into the same subject of a modified ecdysone receptor and, optionally, a silent partner therefore, that binds to the ecdysone response element in the presence of ligand. Thus, those practicing invention methods are not required to perform all the steps that are characteristic of gene therapy.

Even if the present invention were considered to "lie in the realm of gene therapy" as asserted by the Examiner, the Examiner's pessimistic statements regarding the state of gene therapy (see, generally, Office Action dated July 2, 1999, pages 5-7) are unwarranted. Applicants respectfully disagree with the Examiner's assertion that gene therapy is unpredictable due, for example, to problems in predicting transduction efficiency and the outcome of transducted therapeutic genes, relying on Anderson in *Nature* (392:25-30 (1998)). The Anderson reference, however, when read in its entirely, also describes various alternatives available to the artisan, including, for example, choices of vectors for introducing a polynucleotide into a cell (pages 25 to 27). Furthermore, Anderson describes that phase II and phase III gene therapy trials are proceeding, and that the two girls treated using "the original adenosine deaminase deficiency gene therapy trial" in 1990 are well and continue to lead essentially normal lives (page 28). Thus, it is submitted that, when taken as a whole, the Anderson reference discloses that gene therapy has been and can continue to be an efficacious therapy.

Indeed, recent published reports providing details of several successful gene therapy treatments of human subjects further support the position that gene therapy methodology is an effective technique to successfully treat various disease conditions. For example, gene therapy methods were used to treat human subjects having severe combined immunodeficiency (SCID)-Xi disease, and "gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit" (Cavazzana-Calvo *et al.,* (2000) *Science* 288:669-672, 669). The patients, who might otherwise have died from a severe immune disorder (SCID-X1), were infused with $CD34^+$ cells, transfected using *ex vivo* techniques with a retrovirus vector containing a therapeutic gene.

In another positive gene therapy outcome, intramuscular injection into skeletal muscle was used to successfully supply to human haemophiliac patients an adeno-associated viral vector containing the sequence for blood coagulation factor IX (Kay, *et al.*, (2000) *Nature Genetics* 24:257-61). Moreover, in this clinical trial, there was gene expression in human patients following injection with very low doses of vector, leading the scientists to suggest that "dose calculations based on animal data may have overestimated the amount of vector required to achieve therapeutic levels in humans" (*id.*, 257).

Thus, in view of the Anderson reference and supported by recent reports, one skilled in the art would have every reason to believe that gene therapy can be practiced in humans and can provide a therapeutic advantage to the treated subjects. Accordingly, Applicants respectfully submit that invention claims, even if deemed to be in the realm of gene therapy, are fully supported by the specification.

Applicants respectfully request prompt and favorable consideration of the present claims which Applicants believe are in condition for allowance.

If the Examiner would like to discuss any of the issues raised herein, Applicants' representative can be reached at (858) 677-1409.

Respectfully submitted,

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Exhibit A

PATENT
ATTORNEY DOCKET NO. SALK1520-2

Applicant:	Evans et al.	Art Unit:	1633
Application No.:	09/042,488	Examiner	S. Kaushal
Filed:	March 16, 1998		
Title:	METHOD FOR MODULATING EXPRESSION OF EXOGENOUS GENES IN MAMMALIAN SYSTEMS, AND PRODUCTS RELATED THERETO		

EXHIBIT A

1. (Reiterated) A method for modulating the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and

(ii) a modified ecdysone receptor which, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising administering to said subject an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in the cells of said subject; and wherein said ligand is not toxic to said subject.

2. (Reiterated) A method according to claim 1 wherein said modified ecdysone receptor comprises:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain obtained from a DNA-binding protein; and

an activation domain of a transcription factor,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor,

with the proviso that when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an *E. coli* LexA protein.

3. (Reiterated) A method according to claim 2 wherein said modified ecdysone receptor is further characterized as having substantially no constitutive activity in mammalian cells.

4. (Reiterated) A method according to claim 2 wherein the DNA-binding domain of said modified ecdysone receptor is derived from a member of the steroid/thyroid hormone superfamily of receptors.

5. (Reiterated) A method according to claim 2 wherein said activation domain is obtained from a member of the steroid/thyroid hormone superfamily of receptors.

6. (Reiterated) A method according to claim 2 wherein said activation domain is selected from a glucocorticoid receptor activation domain, a VP16 activation domain or a GAL4 activation domain.

7. (Reiterated) A method according to claim 6 wherein said modified ecdysone receptor is selected from VpEcR, VgEcR or GEcR.

8. (Reiterated) A method according to claim 7 wherein said modified ecdysone receptor is VgEcR having the amino acid sequence set forth in SEQ ID NO:5.

9. (Reiterated) A method according to claim 1 wherein said modified ecdysone receptor is present primarily in the form of a homodimer.

10. (Reiterated) A method according to claim 9 wherein said ecdysone response element is the native ecdysone response element.

11. (Reiterated) A method according to claim 1 wherein said receptor capable of acting as a silent partner is RXR.

12. (Reiterated) A method according to claim 11 wherein said RXR is exogenous to said mammalian subject.

13. (Reiterated) A method according to claim 1 wherein said ecdysone response element is a modified response element which comprises, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

wherein said first half-site has the sequence:

-RGBNNM-,

wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

each M is independently selected from A or C;

with the proviso that

at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

14. (Reiterated) A method according to claim 13 wherein said response element has substantially no binding affinity for farnesoid X receptor (FXR).

15. (Reiterated) A method according to claim 1 wherein said ligand is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

16. (Reiterated) A method according to claim 15 wherein said naturally occurring ecdysone is α-ecdysone or β-ecdysone.

17. (Reiterated) A method according to claim 15 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, muristerone A, inokosterone or 26-mesylinokosterone.

18. (Reiterated) A method according to claim 15 wherein said ecdysone mimic is 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine.

19. (Reiterated) A method according to claim 1 wherein said exogenous gene is a wild type gene and/or therapeutic gene.

20. (Reiterated) A method according to claim 19 wherein said wild type gene is selected from genes which encode products:

the substantial absence of which leads to the occurrence of a non-normal state in said subject; or

a substantial excess of which leads to the occurrence of a non-normal state in said subject.

21. (Reiterated) A method according to claim 19 wherein said therapeutic gene is selected from those which encode products:

which are toxic to the cells in which they are expressed; or

which impart a beneficial property to said subject.

22. (Reiterated) A method of inducing the expression of an exogenous gene in a mammalian subject containing:

(i) a DNA construct comprising an exogenous gene under the control of an ecdysone response element,

(ii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, and

(iii) a ligand for said modified ecdysone receptor;

said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

23. (Reiterated) A method of inducing expression of an exogenous gene in a mammalian subject containing a DNA construct containing said exogenous gene under the control of an ecdysone response element, said method comprising introducing into said subject:

a modified ecdysone receptor; and

a ligand for said modified ecdysone receptor,

wherein said receptor, in combination with a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, activating transcription therefrom.

24. (Reiterated) A method for the expression of a recombinant product detrimental to a host organism, said method comprising:

transforming suitable host cells with:

(i) a DNA construct encoding said recombinant product under the control of an ecdysone response element, and

(ii) DNA encoding a modified ecdysone receptor;

growing said host cells in suitable media; and

inducing expression of said recombinant product by introducing into said host cells ligand(s) for said modified ecdysone receptor, and optionally a receptor capable of acting as a silent partner for said modified ecdysone receptor.

25. (Amended) A formulation comprising at least one ecdysteroid and a pharmaceutically acceptable carrier.

26. (Reiterated) A formulation according to claim 25 wherein said pharmaceutically acceptable carrier renders said formulation suitable for oral, topical, nasal, transdermal, intravenous, subcutaneous, intramuscular, intracutaneous, intraperitoneal or intravascular administration.

27. (Reiterated) A formulation according to claim 25 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

28. (Reiterated) A formulation according to claim 27 wherein said naturally occurring ecdysone is α-ecdysone or β-ecdysone.

29. (Reiterated) A formulation according to claim 27 wherein said ecdysone analog is ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, muristerone A, inokosterone or 26-mesylinokosterone.

30. (Reiterated) A formulation according to claim 27 wherein said ecdysone mimic is 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine.

31. (Amended) A formulation consisting essentially of at least one ecdysteroid and a pharmaceutically acceptable carrier.

32. (Reiterated) A formulation according to claim 31 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

33. (Reiterated) A kit comprising at least one ecdysteroid and a pharmaceutically acceptable carrier therefor.

34. (Amended) A kit according to claim 33 wherein said ecdysteroid is a naturally occurring ecdysone, an ecdysone-analog or an ecdysone mimic.

35. (New) A method according to claim 4, wherein said member of the steroid/thyroid hormone superfamily of receptors is selected from: EcR, vitamin D_3 receptor, RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, TRα, TRβ, or ER.

36. (New) A method according to claim 35, wherein the DNA-binding domain of the modified ecdysone receptor is characterized as having a P-box amino acid sequence that differs from the P-box amino acid sequence of the naturally occurring DNA-binding domain.

37. (New) A method according to claim 36, wherein said modified P-box amino acid sequence preferentially binds to a different hormone response element half-site than said naturally occurring P-box amino acid sequence.

38. (New) A method according to claim 37, wherein the DNA-binding domain of said modified ecdysone receptor is derived from EcR and the P-box amino acid sequence is GSCKV (SEQ ID NO:3).

39. (New) A method according to claim 13, wherein said first half-site is obtained from an ecdysone response element and said second half-site is obtained from a hormone response element selected from a glucocorticoid response element, a mineralocorticoid response element, a progesterone response element or an androgen response element.

40. (New) A method according to claim 39, wherein said first half-site is obtained from an ecdysone response element and said second half-site is obtained from a glucocorticoid response element.

41. (New) A method according to claim 40, wherein said first half-site is AGTGCA and said second half-site is TGTTCT.

42. (New) A method according to claim 13, wherein said ecdysone response element has the sequence AGTGCA-N-TGTTCT.

43. (New) A formulation according to claim 27, wherein said composition is effective to activate at least one member of the steroid/thyroid hormone receptor superfamily.

44. (New) A formulation according to claim 43, wherein said member is an ecdysone receptor.

45. (New) A formulation according to claim 44, wherein said formulation acts to modulate gene transcription for a gene maintained under the control of an ecdysone response element.

46. (New) A formulation according to claim 27 wherein said ecdysteroid is 20-hydroxyecdysone.

Human gene therapy

W. French Anderson

Although gene therapy as a treatment for disease holds great promise, progress in developing effective clinical protocols has been slow. The problem lies in the development of safe and efficient gene-delivery systems. This review will evaluate the problems and the potential solutions in this new field of medicine.

The first approved clinical protocol for somatic gene therapy started trials in September 1990[1]. Since then, in just 7½ years, more than 300 clinical protocols have been approved worldwide and over 3,000 patients have carried genetically engineered cells in their body. The conclusions from these trials are that gene therapy has the potential for treating a broad array of human diseases and that the procedure appears to carry a very low risk of adverse reactions; the efficiency of gene transfer and expression in human patients is, however, still disappointingly low. Except for anecdotal reports of individual patients being helped, there is still no conclusive evidence that a gene-therapy protocol has been successful in the treatment of a human disease. Why not?

In this review I will examine the 'why not?' by evaluating the promise and the problems of gene therapy. There are various categories of somatic cell gene therapy, distinguished by the mode of delivery of the gene to the affected tissue (see Box 1). The challenge is to develop gene therapy as an efficient and safe drug-delivery system. This goal is more difficult to achieve than many investigators had predicted 5 years ago. The human body has spent many thousands of years learning to protect itself from the onslaught of environmental hazards, including the incorporation of foreign DNA into its genome. Viruses, however, have been partially successful in overcoming these barriers and being able to insert their genetic material into human cells. Hence the initial efforts at gene therapy have been directed towards engineering viruses so that they could be used as vectors to carry therapeutic genes into patients. A number of reviews on aspects of gene therapy have been published recently[2-10]; this review will consider the categories of the various virus vectors in turn.

Vectors based on RNA viruses

Retroviruses were initially chosen as the most promising gene-transfer vehicles[11]. Currently, about 60% of all approved clinical protocols utilize retroviral vectors. These RNA viruses can carry out efficient gene transfer into many cell types and can stably integrate into the host cell genome (Fig. 1), thereby providing the possibility of long-term expression. They have minimal risk because retroviruses have evolved into relatively non-pathogenic parasites (although there are exceptions, such as the human immunodeficiency viruses (HIV) and human T-cell lymphotropic viruses (HTLV)). In particular, murine leukaemia virus (MuLV) has traditionally been used as the vector of choice for clinical gene-therapy protocols, and a variety of packaging systems to enclose the vector genome within viral particles have been developed. The vectors themselves have all of the viral genes removed, are fully replication-defective and can accept up to about 8 kilobases (kb) of exogenous DNA.

Box 1 The three categories of somatic cell gene therapy

- The first is *ex vivo*, where cells are removed from the body, incubated with a vector, and the gene-engineered cells are returned to the body. This procedure is usually done with blood cells because they are the easiest to remove and return.
- The second is *in situ*, where the vector is placed directly into the affected tissues. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a tumour mass with a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy.
- The third is *in vivo*, where a vector could be injected directly into the blood stream. There are no clinical examples of this third category as yet, but if gene therapy is to fulfil its promise as a therapeutic option, *in vivo* injectable vectors must be developed.

The problems that investigators face in developing retroviral vectors that are effective in treating disease are of four main types: obtaining efficient delivery, transducing non-dividing cells, sustaining long-term gene expression, and developing a cost-effective way to manufacture the vector.

Obtaining efficient delivery. Clinical protocols with retroviral vectors primarily use the *ex vivo* approach. Currently, the cells that are transduced by retroviral vectors are those that possess a high level of the natural MuLV (amphotropic) receptor and are actively dividing at the time of exposure to the vector. Most human cells that can be grown *in vitro* can be transduced, although a few cell types cannot. An important target cell is the primitive haematopoietic stem cell (HSC) because gene transfer into these cells would result in gene-engineered cells for the life of the recipient. However, HSCs have a low level of amphotropic receptor and are poorly transducible[12]. The HSC remains, therefore, an important but elusive target.

The broad range of cell types possessing the amphotropic receptor, known to be a phosphate symport, limits the target-specific utility of these vectors in the *in vivo* approach. Using different viral envelope proteins that recognize different receptors (for example, the vesicular stomatitis virus (VSV)-G protein or the gibbon ape leukaemia virus (GALV) envelope protein) can vary the range of cells that can be transduced, but still does not provide much specificity. The difficulty is that, because retroviral vectors cannot be generated at a high titre (amphotropic vectors appear to be limited to 1×10^7 colony-forming units (CFU) per ml and VSV-G pseudotyped vectors to 1×10^9 CFU per ml), it is not possible to get a large number of vector particles to the desired cell type *in vivo*. The viral particles would bind to many cells they encounter and, therefore, would be diluted out before reaching their target (other issues, such as complement-mediated lysis, will be discussed later). The problem can be quantified. The human body contains about 5×10^{13} cells. If a 100 ml sample of retroviral vector were given to a patient, that would be about 1×10^9 active vector particles. Even if every vector particle were 100% efficient at infection, only 1 cell in 50,000 would be transduced. What is needed is a retroviral particle that will preferentially bind to its target cell and can be manufactured at a high titre.

Efforts to target specific cell types have centred on attempts to engineer the natural retroviral envelope protein. The envelope protein has two functions: binding to its receptor (by the surface (SU) moiety) and enabling the entry of the viral nucleoprotein core (carried out primarily by the transmembrane (TM) moiety). The SU protein binds to its receptor on the target cell surface and, as a result, the SU/TM complex undergoes a conformational change that allows fusion of the viral and cellular membranes, followed by entry of the viral core (which carries the virus's genetic information) into the target cell's cytoplasm (Fig. 1).

Two broad approaches to providing target cell specificity have been followed. First, the natural receptor-binding domain of the SU protein has been replaced with a ligand or single-chain antibody that recognizes a specific cell surface receptor[13,14]. A wide range of receptors have

been targeted, but the difficulty is that even though specific binding can be obtained between the engineered vector and the target cell receptor, gene transfer has been unacceptably low in all these experiments. The reason is clear. The retroviral envelope protein is thought to be a trimer with a complex quaternary structure[15]. When the natural receptor-binding domain is replaced by a foreign sequence, the whole structure of the envelope protein is altered. The result is that the natural post-binding conformational change that leads to the fusion of the virus with the cell membrane does not occur. Without fusion, core entry and gene transfer do not take place efficiently.

Engineering the receptor-binding domain of SU while maintaining the ability of the envelope protein to carry out core entry will require a better understanding of the structure–function relationships within the envelope protein complex. This understanding has been enhanced by the recent publication of the three-dimensional structure of the receptor-binding domain of the murine ecotropic (Friend strain) SU protein[16]. It should now be possible to engineer ligands into very specific sites in the SU protein with a higher probability of maintaining the functional properties of the envelope protein for core entry.

Other structure–function studies of the retroviral envelope protein are also contributing to our understanding of how to obtain efficient core entry after binding. The three-dimensional structure of a portion of the Moloney ecotropic retroviral TM protein was published last year[15]. Recently it has been shown that the separate monomers in the predicted trimeric structure of the envelope can cross-talk with each other[17]. In other words, separate monomers, each of which is defective, can complement each other to provide an active trimeric envelope. Using this technique it has been possible to define separate functional domains in the TM protein[18]. As the complete three-dimensional structure and functional domains of the envelope protein become known, constructing retroviral vectors that are able to target specific cells with high efficiency should be possible.

Progress has been made using a second broad approach to targeting that could be called 'tethering'. Although several creative systems have been designed[7], the most successful approach at present appears to be insertion of a ligand that recognizes an extracellular matrix (ECM) component into a part of the SU protein that does not disturb the natural receptor-binding domain. This tethering concentrates the vector in the ECM in the vicinity of the target cells. Receptor binding and core entry can then occur through the natural envelope-receptor mechanism. Two ligands that appear particularly useful for tethering are those specific for fibronectin[19] and for collagen[20]. Fibronectin is present in normal ECM and exposed collagen is present in areas of damage, for example after wound injury as in the cardiovascular system after angioplasty.

Transduction of non-dividing cells. Although the inability of MuLV-based retroviral vectors to transduce non-dividing cells is very useful in some situations, for example when a toxin gene is being inserted into dividing cancer cells and not into the normal non-dividing cells (see below under 'Clinical studies'), there are many situations where one would want to insert a therapeutic gene into normal non-dividing cells. Many potential target cells are not actively dividing *in vivo*; only certain blood cells (not the stem cell) and the cells lining the gastrointestinal tract are continually in division. Lentiviruses (such as HIV-1) are able to infect non-dividing cells, but vectors constructed from these viruses raise concerns over safety because of the possibility that recombination could produce a pathogenic virus. Attempts to transfer into murine retroviral vectors the specific signals from HIV that allow transduction of non-dividing cells have not been successful. Recently it has become possible to use just 22% of the HIV genome (which does not include any of the genes that cause pathology) in a retroviral vector[21,22]. The chances of recombination have been further reduced by the use of a non-HIV envelope protein. This hybrid system holds great promise for providing the option of transducing non-dividing cells *in vivo* in a safe manner. Another RNA viral system being developed is based on the human foamy virus[23]. These vectors are able to transduce a broad range of cell types, are not inactivated by human serum, and may be able to transduce some non-dividing, as well as dividing, cells.

Improving gene expression. Assuming that efficient gene transfer can be developed, the next issue is long-term, stable gene expression at an appropriate level[6]. This is perhaps the greatest shortcoming of present vectors. Although gene expression is being discussed here under retroviral vectors, the topic applies to gene transfer vectors of all types.

Several factors are involved in maintaining the stable expression of genes after their transfer. First, the regulatory sequences that control gene expression often do not remain active. There is a tendency for the cell to recognize foreign promoters (particularly viral promoters such as simian virus 40 (SV40) and cytomegalovirus (CMV)) and inactivate them (by methylation or other mechanisms). The role of lymphokines, cytokines and other growth factors in maintaining gene expression is also poorly understood. Second, even if the gene stays active within the cell, the cell often dies. The immune system is designed to recognize and eliminate foreign gene products and cells that produce a foreign protein. All viral genes are eliminated from retroviral vectors, and so immune recognition of viral proteins (except for those, such as capsid proteins, that are packaged into the viral particle itself) is not an issue (but see the discussion of adenoviral vectors below). Nonetheless, the immune system is still likely to recognize a new or modified protein produced by the therapeutic gene; a newly synthesized normal protein will appear abnormal to an immune system that has never been exposed to it.

Use of a cell's own *cis*-regulatory DNA sequences will probably provide more stable long-term gene expression than can be obtained with viral promoters, but identifying all the components of a gene's regulatory system can be difficult. As an extreme case, the regulatory sequences involved in the proper regulation of the haemoglobin (β-globin) genes are spread over nearly 100 kb. Because a retroviral vector can only accommodate 6–8 kb of sequence, regulatory sequences may need to be truncated to their minimal essential length before being incorporated into such vectors. Even when the natural regulatory elements are used, they may not function correctly without the proper signals and feedback mechanisms that normally operate in the appropriate cellular milieu. For example, the insulin enhancer/promoter still cannot direct regulated expression when delivered to fibroblasts. Again, this emphasizes the need to develop vectors that are capable of gene transfer to specific cell types.

There is steady progress on these fronts, but long-term, stable, appropriate-level gene expression *in vivo* in a range of cell types is still to be accomplished. Once these hurdles are cleared, the next goal will be to achieve gene expression that can be regulated. Many important target genes, such as that for insulin, are not expressed at the same level all the time, but respond to physiological signals within the body. The goal is to use regulatory sequences that respond to the body's own physiological signals (so that inserted therapeutic genes can function the way that normal endogenous genes do) or to drugs that can be used to control the level of gene activity. In some cases, only low levels of essentially unregulated expression may be beneficial (for example, in haemophilia or adenosine deaminase (ADA) deficiency), whereas in other cases tight regulation may be required (for example, for insulin production in diabetes).

Manufacturing the vector. Although consideration of how a pharmaceutical company would be able to manufacture millions of doses of a gene-therapy vector was irrelevant a decade ago, this has now become a real issue. Retroviral vectors are biological agents: they can only be made by living cells. Biological systems are not the easiest systems in which to carry out good manufacturing practice (GMP) and quality assurance/quality control (QA/QC) procedures mandated by the Food and Drug Administration (FDA), as manufacturers of vaccines have learned.

One of the major concerns with retroviral vectors is the possibility that a replication-competent retrovirus (RCR) could arise during the manufacturing process. Because retroviral vectors are produced in

packaging cells that contain a packaging-defective viral genome, and because retroviruses have a high propensity for recombination, this possibility is always present. Furthermore, as every mammalian cell contains endogenous retroviruses, additional viral sequences could be incorporated into the RCR, perhaps producing a pathogenic virus.

Another potential problem results from the ability of retroviral vectors to integrate randomly into host cell DNA. For example, a vector might insert itself into a tumour suppressor gene, thereby increasing the propensity of the cell to become cancerous. The only example of unintentional tumour production in a retroviral gene transfer experiment in large animals was published in 1992; three cases of lymphoma were reported among ten rhesus monkeys whose bone marrow had been destroyed by irradiation and who were then transplanted with haematopoietic stem cells that had been exposed to a large number of RCR as well as the experimental vector[24]. It was shown that the cancers resulted from integration of an RCR (not of the retroviral vector), were clonal events and developed only after a long period (6–7 months) of retroviraemia.

The subject of RCR production and safety as well as of potential tumour production was extensively analysed in a report to the NIH Recombinant DNA Advisory Committee (RAC) and the FDA[25]. The conclusion was that the current QA/QC procedures required by the FDA make it exceedingly unlikely that any patient could receive sufficient RCR to produce either a retroviraemia or a malignancy. However, the manufacturing and testing process to ensure this degree of safety is complex and expensive.

As the goal of present research is the production of a gene therapy vector that can be injected directly into the body (just like penicillin or insulin), additional problems must be considered. For example, mouse packaging cells produce retroviral vectors that are destroyed by human complement. Although this sensitivity makes the vector particles 'safer', it does markedly reduce their half-life *in vivo* and the efficiency of gene transfer. The major component of this sensitivity arises from the presence of unique sugar groups on viral glycoproteins produced in the murine packaging cells that make the viral particles sensitive to human complement. Either the vector particles produced in mouse cells must be engineered to avoid the human complement system, or the vector needs to be made in a non-murine packaging cell line that can provide the viral particles with appropriate sugar groups on their surface. However, as mentioned above, essentially all mammalian cells have their own endogenous retroviruses that could recombine with the vector to produce a new, potentially pathogenic, RCR; many of these endogenous viruses are still unknown. Although any cell line is suspect, the use of primate or human cells as packaging cells raises the greatest safety concerns in this regard. Human packaging cells can, however, be engineered to be very safe. For example, the ProPak cell line[26], which has the viral *gag–pol* genes on a separate DNA construct from the *env* gene (producing a 'split' packaging cell line) as well as other safety features, is certainly safer than the murine packaging cell line PA317, which is used for most of the present retroviral vector clinical trials.

These issues are resolvable, but it will take several years of product development to develop a cost-effective manufacturing system that will produce safe, efficient gene-therapy vectors on a sufficient scale to allow worldwide marketing. Although a non-viral delivery system that avoids many of these problems may be the gene-therapy vector of the future (see discussion below under 'Non-viral vectors'), the many present and future clinical protocols using retroviral vectors require that the manufacturing issues of safety and efficiency be solved.

Vectors based on DNA viruses

Adenoviral vectors. The DNA virus used most widely for *in situ* gene transfer vectors is the adenovirus (specifically serotypes 2 and 5). Adenoviral vectors have several positive attributes: they are large and can therefore potentially hold large DNA inserts (up to 35 kb, see below); they are human viruses and are able to transduce a large number of different human cell types at a very high efficiency (often reaching nearly 100% *in vitro*); they can transduce non-dividing cells; and they can be produced at very high titres in culture. They have been the vector of choice for several laboratories trying to treat the pulmonary complications of cystic fibrosis, as well as for a variety of protocols attempting to treat cancer.

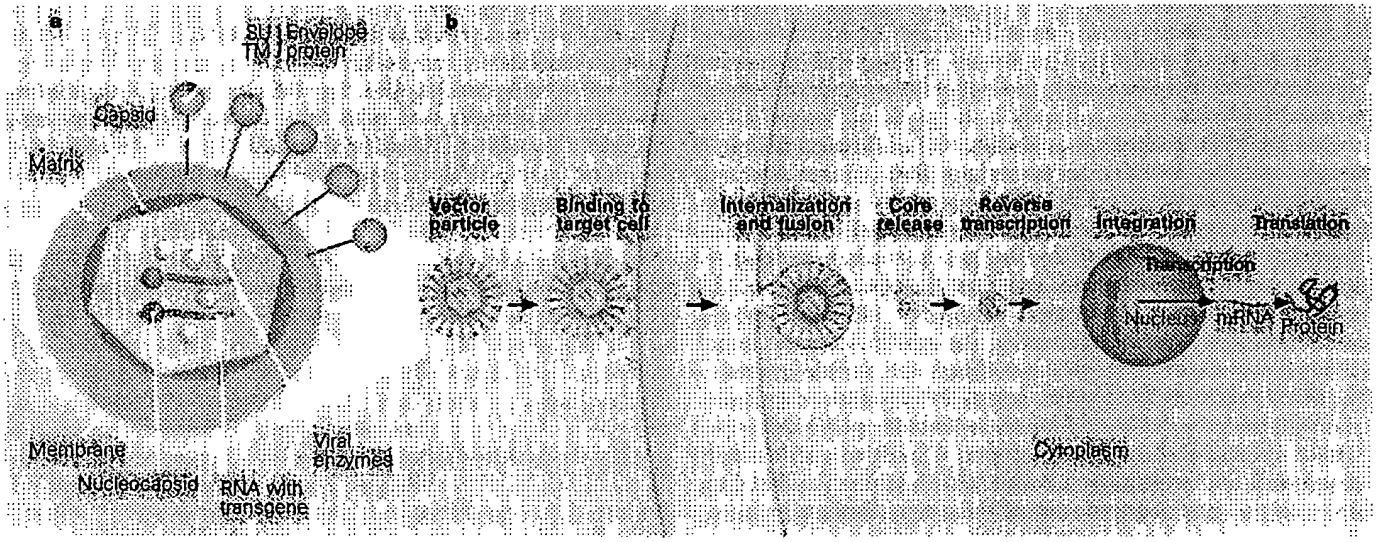

Figure 1 The protocol for retroviral vectors. **a**, Diagram of a retroviral vector. The vector particle is covered by a membrane (derived from the membrane of the cell from which the viral particle budded). Anchored in the membrane is the envelope protein, which is composed of two parts: the SU (surface) protein, which is responsible for binding to the receptor on the target cell, and the TM (transmembrane) protein, which passes through the membrane into the viral matrix and is involved in the fusion step. Beneath the viral membrane is the matrix protein and deeper still is the viral core, which is composed of a surrounding capsid within which are two identical strands of RNA together with the nucleocapsid protein and the viral enzymes (protease, polymerase and integrase). In a retroviral vector the viral genes have been replaced by a transgene. **b**, Diagram of a retroviral vector transducing a target cell. First, the vector particle binds to its receptor on the target cell by means of its SU envelope protein. The particle is then internalized into the cytoplasm of the cell, encased inside an endosome. The envelope protein initiates fusion of the viral membrane with the endosomal membrane, causing the viral core to be released into the cytoplasm. Reverse transcription takes place within the core, which results in the RNA being copied into a double strand of DNA. The double-stranded viral DNA then enters the nucleus, integrates into the chromosomal DNA, and is transcribed. Because the viral genes have been replaced by a transgene, only the protein product of the transgene is made instead of new viral particles.

Adenoviral vectors have certain drawbacks, however. First-generation vectors were deleted for the early region 1 (E1) functions in order to render them replication-defective. In addition, these vectors were deleted in the E3 region in order to create space for the insertion of transgenes. The E3 region, as discussed below, functions to suppress the host immune response during virus infection, but is not required for replication or packaging *in vitro*. Vectors with E1 and E3 deleted elicited strong inflammatory and immune responses[27]. This is thought to be a consequence of 'leaky' expression of adenoviral proteins in the transduced cells because these first-generation vectors retain most of the viral genome. It was hoped that a weaker immune response would result if additional viral genes were deleted. Thus vectors with the deletion of E1 coupled with the deletion of other essential early genes, E2a and/or E4 (refs 28, 29), or vectors with all of the viral genes deleted (so-called 'gutless' vectors[30–32]) have been constructed and tested in animals There have been conflicting reports regarding the immunogenicity, stability of gene expression, and persistence *in vivo* of gutless vectors[33]. In fact, these properties may differ depending on the exact vector design, the tissue type that the vector is introduced into, and the nature of the transgene insert. In particular, the gutted vectors offer the possibility of introducing up to 35 kb of genomic sequences, and it has been suggested that inclusion of nuclear matrix attachment regions might facilitate long-term gene expression and persistence of the vector sequences.

Deleting more and more viral genes may not always be advantageous because some of these genes may have beneficial attributes, for example suppressing an immune response against the vector. Their removal could increase the rate at which the vector is eliminated. As an example, the E3 region encodes a protein of relative molecular mass 19K that protects the virus, and presumably the engineered cells, from host immune surveillance[34]. Various effector mechanisms may be involved in viral vector clearance[35]. In addition, *cis*-acting sequences may exist that help maintain the stability of the adenoviral genome in the cell. As with drug trials, results in animals (even in primates) have not always reflected what happens in patients. Vectors that produce inflammatory responses in primates may not do so in human patients, and the opposite situation is probably also likely. Recently, the first 'true' phase I gene therapy clinical trials have begun: normal volunteers have been tested with intradermal injection (and now by intrabronchial infusion) of adenoviral vectors in order to determine the immunological response to adenoviral vectors in human beings.

By engineering the correct combination of viral genes (incorporating immunosuppressive genes, perhaps from various sources, while deleting immune-stimulating gene products and reducing, if possible, the immunogenicity of viral capsid proteins), it is likely that adenoviral vectors can be generated that have low toxicity, that do not generate an immune response, and that, therefore, can be given repeatedly. The latter point is important because adenoviral vectors do not integrate and they survive in the cell for a limited time (although in non-dividing cells this may be for an extended period). The ability to administer the vector repeatedly will be critical in many treatment protocols, for example in those for haemophilia and cystic fibrosis. Although it would clearly be optimal to engineer vectors that do not elicit an immune response, an interim solution could be to use transient immunosuppression of the patient to allow repeated administration of vectors. Another approach is to blockade costimulatory interactions required for an immune response to an antigen, thereby transiently 'blinding' the immune system during vector administration and making repeat administration possible.

Adeno-associated viral vectors. Another DNA virus used in clinical trials is the adeno-associated virus (AAV). This is a non-pathogenic virus that is widespread in the human population (about 80% of humans have antibodies directed against AAV). Initial interest in this virus arose because it is the only known mammalian virus that shows preferential integration into a specific region in the genome (into the short arm of human chromosome 19). As the virus does not produce disease, its insertion site appears to be a 'safe' region in the genome. It would be useful, therefore, to engineer the sequences that dictate this site-specific insertion into gene-therapy vectors. Unfortunately, the present AAV vectors appear to integrate in a nonspecific manner[36], although it has been suggested that vectors could be designed that retain some specificity[37].

Even though integration site specificity has not been achieved, AAV vectors have been shown to transduce brain, skeletal muscle, liver and possibly CD34$^+$ blood cells efficiently[2,38–40]. There are several drawbacks, however: some cells require a very high multiplicity of infection (the number of viral particles per cell required to achieve transduction); the AAV genome is small, only allowing room for about 4.8 kb of added DNA; and the production of viral particles is still very labour intensive because efficient packaging cells have not yet been developed. However, these vectors hold promise and appear to be safe. Furthermore, AAV may be capable of integrating into non-dividing cells, although again this desirable attribute of the wild-type virus appears to be lost from the vectors, which can enter non-dividing cells but remain in an episomal state until cell division occurs.

Other DNA virus-based vectors

Other DNA viruses are being studied as possible gene-therapy vectors for specific situations. For example, herpes simplex virus (HSV) vectors have a propensity for transducing cells of the nervous system[41,42], as well as several other cell types. A stripped-down version of the HSV, called an amplicon, may have certain advantages, particularly when combined with components from other viral systems[43]. A number of other DNA virus vectors are under study including poxviruses.

Several investigators are examining replication-competent, or attenuated, viral vectors (both DNA and RNA). In addition, hybrid systems have been reported where an adenoviral vector is used to carry a retroviral vector into a cell that is normally inaccessible to retroviral transduction[44].

Non-viral vectors

Although viral systems are potentially very efficient, two factors suggest that non-viral gene delivery systems will be the preferred choice in the future: safety, and ease of manufacturing. A totally synthetic gene-delivery system could be engineered to avoid the danger of producing recombinant virus or other toxic effects engendered by biologically active viral particles. Also, manufacturing a synthetic product should be less complex than using tissue culture cells as bioreactors, and QA/QC procedures should be simplified. The reader is referred to the review on non-viral vectors entitled 'Drug delivery and targeting' by Robert Langer on pages 5–10 of this issue.

Table 1 Disease targets and gene-therapy protocols

(a) Types of gene therapy clinical protocols*

Type	Number	Percentage of total
Therapy	200	(86%)
Marker	30	(13%)
Non-therapeutic†	2	(1%)
Total	232	(100%)

(b) Disease targets for therapeutic gene therapy clinical protocols

Target	Number	Percentage of total
Cancer	138	(69%)
Genetic diseases	33	(16.5%)
CF	16	
Other‡	17	
AIDS	23	(11.5%)
Other§	6	(3%)
Total	200	(100%)

* Roughly 60% of all protocols use retroviral vectors, 20% use non-viral delivery systems, 10% use adenoviral vectors and the remainder use other viral vectors.

† A 'non-therapeutic' protocol means a non-therapeutic portion of a non-gene-therapy clinical protocol.

‡ These 17 include 12 other monogenic diseases.

§ The five 'other' are: peripheral artery disease, rheumatoid arthritis, arterial restenosis, cubital tunnel syndrome and coronary artery disease (2).

Clinical studies

At present over 300 clinical protocols have been approved. Detailed information is available on the 232 protocols that had been approved in the USA as of 3 February 1998[45] (Table 1).

Only one phase III and several phase II clinical trials are now underway; all the rest of the approved gene therapy clinical protocols are for smaller phase I/II trials. Genetic Therapy Inc./Novartis is carrying out the phase III clinical trial. The target disease is glioblastoma multiforma, a malignant brain tumour[46]. The rationale is to insert a gene capable of directing cell killing into the tumour while protecting the normal brain cells. The retroviral vector used (G1TkSvNa) contains the neomycin-resistance gene as a selective marker and the herpes simplex thymidine kinase (HSTk) gene. The actual material injected into the tumour mass is a mouse producer cell line (PA317) which generates retroviral particles carrying the G1TkSvNa vector. As the only dividing cells in the area of a growing brain tumour are the tumour cells and cells of the vasculature supplying blood to the tumour, and retroviral vectors only transduce dividing cells, the only cells to receive the vector should be the cells of the tumour and its blood vessels. The viral HSTk can add a phosphate group to a non-phosphorylated nucleoside, whereas the endogenous human thymidine kinase cannot. Therefore, when an abnormal nucleoside, such as the drug ganciclovir, is given to the patient, only the cells expressing the HSTk gene will phosphorylate the drug, incorporate it into their DNA synthesis machinery and be killed.

In the current phase III clinical trial, mouse producer cells making vector particles carrying the HSTk gene are inoculated into residual tumour and peritumour areas following tumour resection. After 7 days, the patient is treated with ganciclovir[47]. In theory, the tumour cells that have been transduced with the vector containing the HSTk gene will phosphorylate ganciclovir; the ganciclovir triphosphate then blocks the DNA synthesis machinery and kills the cells.

In fact, at least four distinct mechanisms contribute to tumour cell death in this protocol. First is the direct effect of phosphorylated ganciclovir on the transduced tumour cells; second is the 'bystander' effect in which toxic agents (ganciclovir triphosphate) pass into neighbouring cells through gap junctions and kill them; third, is the local inflammatory effect caused by the injected mouse cells; and fourth is a systemic immune response. The phase III trial includes a total of more than 40 centres in North America and Europe and is scheduled to enrol a total of 250 patients. By the end of December 1997 over 200 patients had been enrolled.

Several phase II trials are underway testing gene-therapy vectors as 'vaccines', either against cancer[48] or against AIDS[49]. Vical has two active phase II trials using a plasmid containing the gene for the HLA-B7/β_2-microglobulin protein formulated with cationic lipids. One trial is for metastatic malignant melanoma and the other for head and neck squamous cell carcinoma. The concept is that an HLA gene (such as B7) that the tumour does not express is injected into the tumour mass and that expression of this foreign antigen should stimulate the immune system to react against the cancer. The data so far suggest that the immune system not only develops a response against the B7 antigen but also to other antigens on the tumour cells, thereby resulting in an immune attack on non-transduced tumour cells[50]. Viagen/Chiron has completed a phase II trial of about 200 patients over 2 years in which a retroviral vector encoding the *env* and *rev* gene segments of the HIV-1(IIIB) strain is injected intramuscularly to induce augmented anti-HIV cytotoxic T-cell responses as a treatment for AIDS. Unfortunately, determination of the efficacy of this treatment was made impossible by the advent of triple drug therapy for HIV infection, but no evidence of toxicity was seen.

Finally, a comment on the original adenosine deaminase (ADA) deficiency gene-therapy trial[1,51]. ADA deficiency is a rare genetic disorder that produces severe immunodeficiency in children. Starting in 1990, gene-corrected autologous T lymphocytes were given to two girls suffering from this disease. Both girls are doing well and continue to lead essentially normal lives. Patient 1 (A.D.) received a total of 11 infusions, the last being in the summer of 1992. Her total T-cell level and her level of transduced T cells have remained essentially constant for the past 5½ years. She contracted chickenpox in late 1996 and experienced the same clinical course as would have been expected for any normal 10-year-old. Both she and patient 2 (C.C.) continue to receive polyethylene glycol (PEG)-ADA. Although both girls have gene-engineered T lymphocytes in their circulation after more than 7 years, no definitive conclusion can be drawn as to the relative roles of PEG-ADA and gene therapy in their excellent clinical course.

Ethical issues

Somatic cell gene therapy for the treatment of serious disease is now accepted as ethically appropriate. Indeed, it is so well accepted, and the side effects from gene-therapy protocols have been so minimal, that the danger now exists that genetic engineering may be used for non-disease conditions, that is for functional enhancement or 'cosmetic' purposes. The first Gene Therapy Policy Conference organized by the NIH RAC focused on this issue in September 1997. The conclusion was that enhancement engineering is about to take place, and could slip through the regulatory process if RAC and the FDA (and similar organizations in other countries) are not vigilant. As an example, a US biotechnology company has developed the technology for transferring genes (specifically the tyrosinase gene) into hair follicle cells[52]. They are now looking for genes that promote hair growth with the clinical objective of reversing the hair loss that occurs after chemotherapy in cancer patients. The application to the FDA for product licensing would list chemotherapy-induced alopecia as the product indication. The risk–benefit analysis here would be very favourable. However, once a product is licensed for any indication, it can be prescribed by physicians for any 'off-label' use that is felt by the physician to be clinically justified. The result could be millions of balding men receiving gene therapy to treat their hair loss. The conference concluded that the FDA should use a risk–benefit analysis that takes into account the extensive off-label usage for cosmetic reasons that could take place.

Using genetic engineering to treat baldness is not a major issue in itself, of course. But this is just one example of how our society is moving towards a slippery slope where genetic engineering might very well be used for a broad range of enhancement purposes, including larger size from a growth hormone gene, increased muscle mass from a dystrophin gene and so on. If we knew that there would be no long-term negative effects of genetic engineering, then widespread, or even frivolous, use of genetic engineering technology might not be detrimental. But just as with nuclear energy, pesticides and fluorocarbons, we as a society tend to see the benefits but are caught off guard by the bad effects of our powerful new technologies. What society wants to do 100 years from now with regards to genetic engineering is their business, but it is our duty to begin the era of genetic engineering in as responsible a manner as possible. Until we have learned about the long-term effects of somatic cell gene therapy in the treatment of disease, we should not use this technology for any other purpose than where it is medically indicated[53].

In utero somatic gene therapy of the fetus will be undertaken in the foreseeable future. The same care should be exercised here as with somatic cell gene-therapy protocols for adults, children and newborns. So long as only serious disease is targeted and the risk–benefit ratios for both mother and the fetus are acceptable, *in utero* gene therapy should be ethically appropriate[54]. Germline gene therapy should not be attempted at this time for the reasons outlined elsewhere[55].

A situation with the potential for real abuse of the new technologies would be the combination of cloning and genetic engineering. This combination has already been achieved in sheep where single cells have been obtained from fetal fibroblasts, transduced with a gene (human factor IX), and the gene-engineered cells grown into living sheep producing human factor IX[56]. Attempts to use such techniques to produce genetically engineered humans would provoke an even greater ethical storm than the present suggestion by a Chicago scientist to clone humans.

The future

The ultimate goal of gene-therapy research is the development of vectors that can be injected, will target specific cells, will result in safe and efficient gene transfer into a high percentage of those cells, will insert themselves into appropriate regions of the genome (or will persist as stable episomes), will be regulated either by administered agents or by the body's own physiological signals, will be cost-effective to manufacture and will cure disease. As the number of target cells may be in the billions, very high efficiency of gene transfer and the injection of a large number of gene-therapy vectors may be necessary. How soon can we expect significant progress in each of these areas?

The next 5 years should bring the first successes for gene therapy, that means statistically significant data that a gene-therapy protocol results in significant improvement in the clinical condition of patients. Within this time frame the first vectors that can target specific tissues should begin clinical trials and tissue-specific gene expression should have made its way into clinical trials.

In a time frame of 5–15 years from now, I expect that the number of gene-therapy products will begin to increase exponentially, coinciding with the enormous increase in characterized genes as a result of the Human Genome Project. The first injectable vectors will reach clinical trials and efficient tissue-specific gene transfer will be available in a few cases. It will probably take longer to develop site-specific integration, efficiently regulated genes and the correction of genes *in situ* by means of homologous recombination. Beyond this, our imagination is the limit.

For many gene-therapy applications in the future, it is probable that a synthetic hybrid system will be used that incorporates engineered viral components for target-specific binding and core entry, immunosuppressive genes from various viruses and some mechanism that allows site-specific integration, perhaps utilizing AAV sequences or an engineered retroviral integrase protein. In addition, regulatory sequences from the target cell itself will be utilized to allow physiological control of expression of the inserted genes. All these components would be assembled *in vitro* in a liposome-like formulation with additional measures taken to reduce immunogenicity such as concealment by PEG.

Conclusions

Gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease. Several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered. The reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basic understanding of how vectors should be constructed, what regulatory sequences are appropriate for which cell types, how *in vivo* immune defences can be overcome, and how to manufacture efficiently the vectors that we do make. It is not surprising that we have not yet had notable clinical successes. Nonetheless, the lessons we are learning in the clinic are invaluable in illuminating the problems that future research must solve.

Despite our present lack of knowledge, gene therapy will almost certainly revolutionize the practice of medicine over the next 25 years. In every field of medicine, the ability to give the patient therapeutic genes offers extraordinary opportunities to treat, cure and ultimately prevent a vast range of diseases that now plague mankind. □

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Gene therapy — promises, problems and prospects

Inder M. Verma and Nikunj Somia

In principle, gene therapy is simple: putting corrective genetic material into cells alleviates the symptoms of disease. In practice, considerable obstacles have emerged. But, thanks to better delivery systems, there is hope that the technique will succeed.

In 1990, the first clinical trials for gene-therapy approaches to combat disease were carried out. Conceptually, the technique involves identifying appropriate DNA sequences and cell types, then developing suitable ways in which to get enough of the DNA into these cells. With efficient delivery, the therapeutic prospects range from tackling genetic diseases and slowing the progression of tumours, to fighting viral infections and stopping neurodegenerative diseases. But the problems — such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions — remain formidable challenges.

Although more than 200 clinical trials are currently underway worldwide, with hundreds of patients enrolled, there is still no single outcome that we can point to as a success story. To explore why this is the case, we will use our own experience and other examples to look at the many technical, logistical and, in some cases, conceptual hurdles that need to be overcome before gene therapy becomes routine practice in medicine.

At present, gene therapy is being contemplated only on somatic (essentially, non-reproductive) cells. Although many somatic tissues can receive therapeutic DNA, the choice of cell usually depends on the nature of the disease. Sometimes a clear definition of the target cell is needed. For example, the gene that is defective in cystic fibrosis has been identified, and clinical trials to deliver DNA as an aerosol into the lung have already begun[1]. Although cystic fibrosis is manifest in this organ, it is still not clear that delivery of a correcting gene by this method will reach the right type of cell. On the other hand, to correct blood-clotting disorders such as haemophilia, all that is needed is a therapeutic level of clotting protein in the plasma[2]. This protein may be supplied by muscle or liver cells, fibroblasts, or even blood cells[3–5]. The choice of tissue in which to express the therapeutic protein will also ultimately depend on considerations such as the efficiency of gene delivery, protein modifications, immunological status, accessibility and economics.

We also need to consider how much of the therapeutic protein should be delivered. In haemophilia B, which is caused by a deficiency of a blood-clotting protein called factor IX, giving patients just 5% of the normal circulating levels of this protein can substantially improve their quality of life[2]. Most people have about 5 μg of factor IX per millilitre of plasma, produced by the 10^{13} cells that make up the liver. So we need to deliver a correcting gene to 5×10^{11} cells — that is, 5% of liver cells. Alternatively, fewer liver cells would need to be modified if more factor IX could be produced per cell, without being deleterious. In the brain, however, gene transfer to just a few hundred cells could considerably benefit patients with neurological disease. And finally, we can consider the transfer of genes to a handful of stem (or progenitor) cells, which grow and divide to generate millions of progeny. The range in the number of cells that this technology has to cover is vast.

The Achilles heel of gene therapy is gene delivery, and this is the aspect that we will concentrate on here. Thus far, the problem has been an inability to deliver genes efficiently and to obtain sustained expression. There are two categories of delivery vehicle ('vector'). The first comprises the non-viral vectors, ranging from direct injection of DNA to mixing the DNA with polylysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from poor efficiency of delivery and transient expression of the gene[6]. Although there are reagents that increase the efficiency of delivery, transient expression of the transgene is a conceptual hurdle that needs to be addressed.

Most of the current gene-therapy approaches make use of the second category — viral vectors. Importantly, the viruses used have all been disabled of any pathogenic effects. The use of viruses is a powerful technique, because many of them have evolved a specific machinery to deliver DNA to cells. However, humans have an immune system to fight off the virus, and our attempts to deliver genes in viral vectors have been confronted by these host responses. ▶

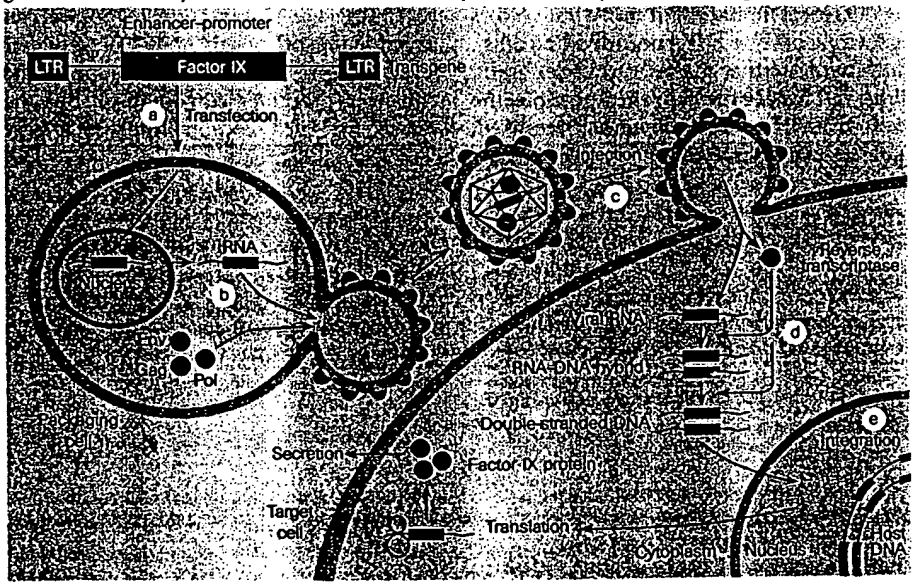


Figure 1 To create the retroviral vectors that are used in gene therapy, the life-cycles of their naturally occurring counterparts are exploited. a, The transgene (in this case, the gene for factor IX) in a vector backbone is put into a packaging cell, which expresses the genes that are required for viral integration (*gag*, *pol* and *env*). b, The transgene is incorporated into the nucleus, where it is transcribed to make vector RNA. This is then packaged into the retroviral vector, which is shed from the packaging cell. c, The vector is delivered to the target cell by infection. The membrane of the viral vector fuses with the target cell, allowing the vector RNA to enter. d, The virally encoded enzyme reverse transcriptase converts the vector RNA into an RNA–DNA hybrid, and then into double-stranded DNA. e, The vector DNA is integrated into the host genome, then the host-cell machinery will transcribe and translate it to make RNA and, in this case, factor IX protein. LTR, long terminal repeat; ψ, packaging sequence.

Retroviral vectors

Retroviruses are a group of viruses whose RNA genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag*, *pol* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome, and they define the beginning and end of the viral genome. The LTRs also serve as enhancer–promoter sequences — that is, they control expression of the viral genes. The final element of the genome, called the packaging sequence (ψ), allows the viral RNA to be distinguished from other RNAs in the cell (Fig. 1)[7].

By manipulating the viral genome, viral genes can be replaced with transgenes — such as the gene for factor IX (Table 1). Transcription of the transgene may be under the control of viral LTRs or, alternatively, enhancer–promoter elements can be engineered in with the transgene. The chimaeric genome is then introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag*, *pol* and *env* genes), but these have been separated from the LTRs and the packaging sequence. So, only the chimaeric viral genomes are assembled to generate a retroviral vector. The culture medium in which these packaging cells have been grown is then applied to the target cells, resulting in transfer of the transgene. Typically, a million target cells on a culture dish can be infected with one millilitre of the viral soup.

A critical limitation of retroviral vectors is their inability to infect non-dividing cells[8], such as those that make up muscle, brain, lung and liver tissue. So, when possible, the cells from the target tissue are removed, grown *in vitro*, and infected with the recombinant retroviral vector. The target cells producing the foreign protein are then transplanted back into the animal. This procedure has been termed '*ex vivo* gene therapy' and our group has used it to infect mouse primary fibroblasts or myoblasts (connective-tissue and muscle precursors, respectively) with retroviral vectors producing the factor IX protein. But within five to seven days of transplanting the infected cells back into mice, expression of factor IX is shut off[3,5,9]. This transcriptional shut-off has even been observed in mice lacking a functional immune system (nude mice), and it cannot be due to cell loss or gene deletion[5] because the transplanted cells can be recovered.

What is the mechanism of this unexpected but intriguing problem? We do not yet know, but the exceptions may provide some clues. To obtain sustained expression in mouse muscle following the transplantation of infected myoblasts, we used the muscle creatine kinase enhancer–promoter to control transcription of the transgene. Unfortunately, this is a weak promoter, and only low levels of protein were produced. So, we generated a chimaeric vector in which the muscle creatine kinase enhancer was linked to a strong promoter from cytomegalovirus. Using this vector, sustained and high levels of factor IX were expressed when the infected myoblasts were transplanted back into mice. Remarkably, these expression levels remained unchanged for more than two years (the life of the animal). So we can override the 'off switch' and achieve higher levels of expression by using an appropriate enhancer–promoter combination. But the search for such combinations is a case of trial and error for a given type of cell.

Another formidable challenge to the *ex vivo* approach is the efficiency of transplantation of the infected cells. Attempts to repeat the long-term myoblast transplantation in haemophiliac dogs led to only short-term expression, because the infected dog myoblasts could not fuse with the muscle fibres. So perhaps successful animal models will prove inadequate when the same protocols are extended to humans. Moreover, these models are based on inbred animals — the outbred human population, with individual variation, will add yet another degree of complexity. The haematopoietic (blood-producing) system may offer an advantage for *ex vivo* gene therapy because resting stem cells can be stimulated to divide *in vitro* using growth factors and the transplantation technology is well established. But there is still a lack of good enhancer–promoter combinations that allow sustained production of high levels of protein in these cells.

Another problem is the possibility of random integration of vector DNA into the host chromosome. This could lead to activation of oncogenes or inactivation of tumour-suppressor genes. Although the theoretical probability of such an event is quite low, it is of some concern (see section on clinical trials).

Lentiviral vectors

Lentiviruses also belong to the retrovirus family, but they can infect both dividing and non-dividing cells[10]. The best-known lentivirus is the human immunodeficiency virus (HIV), which has been disabled and developed as a vector for *in vivo* gene delivery. Like the simple retroviruses, HIV has the three *gag*, *pol* and *env* genes, but it also carries genes for six accessory proteins termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*[11].

Using the retrovirus vectors as a model, lentivirus vectors have been made, with the transgene enclosed between the LTRs and a packaging sequence[12]. Some of the accessory proteins can be eliminated without affecting production of the vector or efficiency of infection. The *env* gene product would restrict HIV-based vectors to infecting only cells that express a protein called CD4⁺ so, in the vectors, this gene is substituted with *env* sequences from other RNA viruses that have a broader infection spectrum (such as glycoprotein from the vesicular stomatitis virus). These vectors can be produced — albeit on a small scale at the moment — at concentrations of $>10^9$ virus particles per ml (ref.12).

When lentivirus vectors are injected into rodent brain, liver, muscle, eye or pancreatic-islet cells, they give sustained expression for over six months — the longest time tested so far[13,14]. Unlike the prototypical retroviral vectors, the expression is not subject to 'shut off'. Little is known about the possible immune problems associated with lentiviral vectors, but injection of 10^7 infectious units

Table 1 **Candidate diseases for gene therapy**

Disease	Defect	Incidence	Target cells
Genetic			
Severe combined immunodeficiency	Adenosine deaminase (ADA)	1 in 200,000 births; 20% of SCID patients have ADA deficiency	Bone-marrow cells or lymphocytes
Haemophilia	A: Factor VIII deficiency	1 in 10,000 males	Liver, muscle fibroblasts or bone-marrow cells
	B: Factor IX deficiency	1 in 30,000 males	
Familial hypercholesterolaemia	Low-density lipoprotein (LDL) receptor	1 in 500 (heterozygotes); 1 in 1 million (homozygotes)	Liver
Cystic fibrosis	Faulty transport of salt ions	1 in 1,000 Caucasians	Airways in the lungs
Haemoglobinopathies	Structural defects or reduced production of globin genes	1 in 600 in certain ethnic groups	Bone-marrow cells giving rise to red blood cells
Gaucher's disease	Defect in the enzyme glucocerebrosidase	1 in 450 in Ashkenazi Jews	Bone-marrow cells, macrophages
Antitrypsin deficiency/inherited emphysema	Lack of α1-antitrypsin	1 in 3,500	Lung or liver cells
Acquired			
Cancer	Many causes (including genetic and environmental)	6 million/year in USA	Variety of cancer cell types: liver, brain, pancreas, breast, kidney
Neurological diseases	Parkinson's, Alzheimer's, spinal cord injury	1 million Parkinson's and 4 million Alzheimer's patients in USA	Direct injection in the brain, neurons, glial cells, Schwann cells
Cardiovascular	Restinosis, arteriosclerosis	13 million in USA	Arteries, vascular endothelial cells
Infectious diseases	AIDS, hepatitis B	Increasing numbers	T cells, liver, macrophages

does not elicit the cellular immune response at the site of injection. Furthermore, there seems to be no potent antibody response. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression.

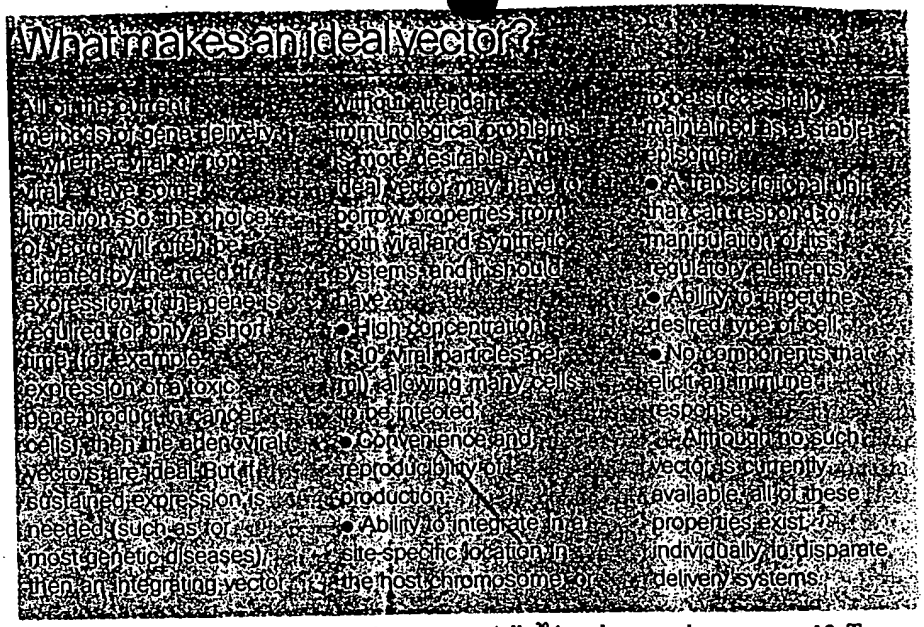
Adenoviral vectors

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory-tract infections in humans[11]. Their genomes contain over a dozen genes, and they do not usually integrate into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of the host cell. Replication-deficient adenovirus vectors can be generated by replacing the *E1* gene — which is essential for viral replication — with the gene of interest (for example, that for factor IX) and an enhancer–promoter element. The recombinant vectors are then replicated in cells that express the products of the *E1* gene, and they can be generated in very high concentrations ($>10^{11}$–10^{12} adenovirus particles per ml)[15].

Cells infected with recombinant adenovirus can express the therapeutic gene but, because essential genes for viral replication are deleted, the vector should not replicate. These vectors can infect cells *in vivo*, causing them to express very high levels of the transgene. Unfortunately, this expression lasts for only a short time (5–10 days post-infection). In contrast to the retroviral vectors, long-term expression can be achieved if the recombinant adenoviral vectors are introduced into nude mice, or into mice that are given both the adenoviral vector and immunosuppressing agents[16]. This indicates that the immune system is behind the short-term expression that is usually obtained from adenoviral vectors.

The immune reaction is potent, eliciting both the cell-killing 'cellular' response and the antibody-producing 'humoral' response. In the cellular response, virally infected cells are killed by cytotoxic T lymphocytes[16,17]. The humoral response results in the generation of antibodies to adenoviral proteins, and it will prevent any subsequent infection if the animal is given a second injection of the recombinant adenovirus. Unfortunately for gene therapy, most of the human population will probably have antibodies to adenovirus from previous infection with the naturally occurring virus.

It is possible that the target cell contains factors that might trigger the synthesis of adenoviral proteins, leading to an immune response. To try to get around this problem, second-generation adenoviral vectors were developed, in which additional genes that are implicated in viral replication were deleted. These vectors showed longer-term expression, but a decline after 20–40 days was still apparent[18]. This idea has now been taken further with the generation of 'gut-less' vectors — all of the viral genes are deleted, leaving only the elements that define the beginning and the end of the genome, and the viral packaging sequence. The transgenes carried by these viruses were expressed for 84 days[19].



There are considerable immunological problems to be overcome before adenoviral vectors can be used to deliver genes and produce sustained expression. The incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction by cytotoxic T cells. So adenoviral vectors are extremely useful if expression of the transgene is required for short periods of time. One promising approach is to deliver large numbers of adenoviral vectors containing genes for enzymes that can activate cell killing, or immunomodulatory genes, to cancer cells. In this case, the cellular immune response against the viral proteins will augment tumour killing. Finally, although immunosuppressive drugs can extend the duration of expression, our goal should be to manipulate the vector and not the patient.

Adeno-associated viral vectors

A relative newcomer to the field, adeno-associated virus (AAV) is a simple, non-pathogenic, single-stranded DNA virus. Its two genes (*cap* and *rep*) are sandwiched between inverted terminal repeats that define the beginning and the end of the virus, and contain the packaging sequence[20]. The *cap* gene encodes viral capsid (coat) proteins, and the *rep* gene product is involved in viral replication and integration. AAV needs additional genes to replicate, and these are provided by a helper virus (usually adenovirus or herpes simplex virus).

The virus can infect a variety of cell types, and — in the presence of the *rep* gene product — the viral DNA can integrate preferentially[20] into human chromosome 19. To produce an AAV vector, the *rep* and *cap* genes are replaced with a transgene. Up to 10^{11}–10^{12} viral particles can be produced per ml, but only one in 100–1,000 particles is infectious. Moreover, preparation of the vector is laborious and, due to the toxic nature of the *rep* gene product and some of the adenoviral helper proteins, we currently have no packaging cells in which all of the proteins can be stably provided. Vector preparations must also be carefully separated from any contaminating adenovirus, and AAV vectors can accommodate only 3.5–4.0 kilobases of foreign DNA — this will exclude larger genes. Finally, we need more information about the immunogenicity of the viral proteins, especially given that 80% of the adult population have circulating antibodies to AAV. These considerations notwithstanding, AAV vectors containing human factor IX complementary DNA have been used to infect liver and muscle cells in immunocompetent mice. The mice produced therapeutic amounts of factor IX protein in their blood for over six months[21,22], confirming the promise of AAV as an *in vivo* gene-therapy vector.

Other vectors

Among the other viruses being considered and developed, is herpes simplex virus, which infects cells of the nervous system[23]. The virus contains more than 80 genes, one of which (*IE3*) can be replaced to create the vector. Around 10^{8}–10^{9} viral particles are produced per ml, but the residual proteins are toxic to the target cell. Additional genes can be deleted, allowing more than one transgene to be included. But if essentially all of the viral proteins are deleted (gut-less vectors), only around 10^{6} viral particles are produced per ml. And, again, many people have an immunity to components of herpes simplex virus, having already been infected at some time.

Vaccinia-virus-based vectors have also

been explored, largely for generating vaccines[24]. The Sindbis and Semliki Forest virus is being exploited as a possible cytoplasmic vector[25] which does not integrate into the nucleus. Although most of these systems produce the foreign protein only transiently, they add diversity to the available systems of gene transfer (Table 2).

Clinical trials

Over half of the 200 clinical trials that have been launched in the United States involve therapeutic approaches to cancer. Nearly 30 of them involve correction of monogenic diseases (Table 1) such as cystic fibrosis, α_1-antitrypsin deficiency and severe combined immunodeficiency (SCID). Most of the trials are phase I (safety) studies and, by and large, the existing delivery systems have no major toxicity problems. Moreover, lessons can be learned from previous clinical trials[26,27]. For example, seven years ago two patients were enrolled in a trial to correct deficiencies in adenosine deaminase (ADA, which leads to severe immunodeficiency). One of the patients improved, and makes 25% of normal ADA in her T cells, five years after the last infusion of infected T cells (although she is still treated with PEG–ADA; bovine adenosine deaminase mixed with polyethylene glycol). But in the other patient, the infected T cells could not produce enough of the deficient enzyme.

The efficacy of gene therapy cannot be evaluated until patients are completely taken off alternative treatments (in the above example, PEG–ADA). In another trial[28], weaning a patient away from PEG–ADA reduced the ability of the T cells to respond *in vitro* to a challenge by pathogens. Clearly, in these cases the retroviral vectors were not optimal, and the number of infected blood-progenitor cells was extremely low. However, these trials did help to establish the technology for generating clinical-grade recombinant retroviral particles, the procedures for infection and transplantation, and the protocols for monitoring patients and analysing data. The disappointing outcome probably lies in the inefficient gene-delivery system. We need a system in which the vector — containing the ADA gene — is efficiently delivered to progenitor cells, leading to sustained expression of high levels of the ADA protein. But, encouragingly, despite being repeatedly injected with highly concentrated recombinant viruses, the patients have suffered no untoward effects to date.

Future prospects

We now need a renewed emphasis on the basic science behind gene therapy — particularly the three intertwined fields of vectors, immunology and cell biology.

All of the available viral vectors arose from understanding the basic biology of the structure and replication of viruses. Clearly, existing vectors need to be streamlined further (see box on page 241), and vectors that target specific types of cell are being developed. The use of antibody fragments, ligands to cell-specific receptors, or chemical modifications to the vector need to be explored systematically. And advances such as the report — published only last week[29] — of the three-dimensional structure of the Env protein from mouse leukaemia virus (a commonly used vector), will facilitate the design of targeted vectors. A better understanding of gene transcription will allow us to design regulatory elements that permit promoter activity to be modulated, and development of tissue-specific enhancer–promoter elements should be vigorously pursued. Finally, we need to begin merging some of the qualities of viral vectors with non-viral vectors, to generate a safe and efficient gene-delivery system.

With respect to immunology, viruses still have many secrets to be unravelled. Viral systems that have evolved to escape immune surveillance can be incorporated into viral vectors. Some of these are being characterized; for example, the adenoviral E3 protein, the herpes simplex ICP47 protein and the cytomegalovirus US11 protein[30]. Systems from other pathogens may also be borrowed and incorporated into vectors. In some cases, the correcting protein will be sensed as foreign, eliciting an immune response. Animal models should help us to understand this and, where necessary, to develop strategies for tolerance.

Cell biology is involved because, in many cases, the goal of gene therapy is to correct differentiated cells, such as epithelial cells in cystic fibrosis and lymphoid cells in ADA deficiency. However, because these cells are continuously replaced there has to be either continued therapy or an attempt to target the stem cells. We first need to develop further the technologies for identifying and isolating these cells, to understand their regulation, and to target infection to them *in vivo*.

So how far have we come since clinical trials began? The promises are still great, and the problems have been identified (and they are surmountable). But what of the prospects? Our view is that, in the not too distant future, gene therapy will become as routine a practice as heart transplants are today. □

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Table 2 Comparison of properties of various vector systems*

Features	Retroviral	Lentiviral	Adenoviral	AAV	Naked/Lipid-DNA
Maximum insert size	~7.5 kb	~7.5 kb	~30 kb	~3.5–4.0 kb	Unlimited size
Concentrations (viral particles per ml)	10^6–10^7	10^6–10^7	10^{11}–10^{13}	10^{11}–10^{12}	No limitation
Route of gene delivery	Ex/in vivo	Ex/in vivo	Ex/in vivo	Ex/in vivo	Ex/in vivo
Integration	Yes	Yes	No	Yes/No	Very poor
Duration of expression in vivo	Long	Long	Short	Long	Short
Stability	Good	Not tested	Good	Good	Very good
Ease of preparation (scale up)	Pilot scale up to 20–50 l	Not known	Easy to scale up	Difficult to purify, difficult to scale up	Easy to scale up
Immunological problems	Few	Few	Extensive	Not known	None
Pre-existing host immunity	Unlikely	Unlikely, except maybe AIDS patients	Yes	Yes	No
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Inflammatory response, toxicity	Inflammatory response, toxicity	None

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Gene therapy

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ABSTRACT **In recent years, there have been a number of technological breakthroughs that have allowed for clinical trials in gene therapy to be initiated. In combination with the genome initiative, the potential for new therapeutics is limitless. Although an enormous amount of information has been obtained in a relatively short period of time, gene therapy is not yet ready for wide-scale practice. Some of the successes and obstacles that remain are summarized in this report.**

Gene Therapy: The Problem

Gene therapy can be defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease. In general, with some exceptions, the nucleic acids are DNA molecules encoding gene products or proteins. The original ideas were directed toward treating monogenic (single-gene) disorders, but it has become clear that the gene can be considered a new pharmaceutical agent for treating many types of diseases. Over the last 20 years, the initial thoughts of gene therapy have been transformed into reality with more than 175 clinical trials and 2,000 patients already treated (1). Yet with all the trials, there is still no conclusive evidence for efficacy. Although the expectations have exceeded the initial success of this relatively new field, important information has been gained from preclinical and clinical trials. With this in mind, it is imperative to realize that with recent technological advances, gene therapy for treating a wide variety of diseases is likely to become a reality within the early part of the next century.

There are clearly a number of obstacles limiting successful gene therapy, but the most difficult to overcome has been the inability to transfer the appropriate gene into a target, non-germ-cell tissue, such that an appropriate amount of gene product (usually a protein) is produced to correct the disease. The cell/organism has developed powerful mechanisms to avoid the accumulation of extraneous genetic material. The purpose of this session was to illustrate both the highlights as well as hurdles that remain for developing successful clinical applications.

Vehicles for Gene Transfer

There are two major classes of vehicles for gene transfer: viral and nonviral vectors. This report will concentrate on vectors that have been used in clinical trials (see Table 1). Some researchers believe that viruses will be most successful because they have evolved for millions of years to become efficient vesicles for transferring genetic material into cells, whereas others believe that some of the side-effects of viruses and possible previous exposures rendering the host resistant to transduction (gene transfer into the cell) will preclude their long-term use in gene therapy. Once a vector is designed, two general approaches are used for gene transfer: *ex vivo* where cells are removed, genetically modified, and transplanted back into the same recipient, and *in vivo* therapy accomplished by transfer of genetic materials directly into the patient. The latter is preferable in most situations, because the complexity of the former method makes it less feasible for wide-scale application.

Viral Vectors for Gene Therapy

Three different classes of viral vectors have been used in clinical trials. The first relates to recombinant retroviruses. To date, retroviruses based on the Mouse Moloney Leukemia virus have been used most frequently in clinical trials. These vectors are packaged into viral particles, have all viral genes removed but contain some of the viral regulatory sequences, and will only transduce dividing cells. Although efficient at transduction into cells in culture, most cells *in vivo* are quiescent at any point in time, making this vector less useful for *in vivo* therapies unless the cells in a target organ are stimulated to cycle. A second disadvantage of retroviruses is the relatively low concentration of virus that can be easily produced. More recently, a chimeric Moloney–Human lentiviral (HIV) vector has been constructed that can transduce at least some quiescent cells *in vivo* including neurons in the brain of rodents (2). This promising advance will require further studies to determine the vector's application in the clinic.

In general, human adenoviruses are responsible for mild illnesses such as upper respiratory infections. The more common serotypes (2 and 5) have been exploited for use in clinical trials for cystic fibrosis and cancer. This 36-kb double-stranded DNA virus contains genes that express more than 50 gene products throughout its life cycle. By eliminating the E1 region of the vector, two goals are accomplished: space is made for placing therapeutic expression sequences and, in the absence of the transactivating E1a protein, the virus cannot replicate. Thus, after gene transfer, no viral spread will occur. These first-generation adenoviral vectors were demonstrated to be very efficient at transferring genes into most tissues after *in vivo* administration. In contrast to retroviruses, these vectors can be concentrated to high titer (10e12/ml), can transduce nondividing cells, and do not integrate into host chromosomal DNA. As a result, these vectors were used to temporarily cure a number of animal models with specific genetic diseases like hemophilia (3) and hypercholesterolenemia (4, 5). Enthusiasm for these vectors was tempered by the discovery that low-level production of viral antigens from the vector elicited a robust immunologic response that eliminated transduced cells and transgene product and/or inhibited repeat administration. These studies have prompted a plethora of studies related to vector–host interactions with the hope that the immunologic barriers could be overcome. Several approaches currently are being pursued. One includes deleting the viral genes that are the most immunogenic, and, more recently, clever methods have been devised to remove all or most of the viral genes (6–8).

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Table 1. Gene transfer vehicles

Vector	Advantages	Disadvantages
Viral		
Retrovirus	Integration into host DNA	Semi-random integration
	All viral genes removed	Transduction requires cell division
	Relatively safe	Relatively low titer
Adenovirus	Higher titer	Toxicity
	Efficient transduction of nondividing cells *in vitro* and *in vivo*	Immunological response
		Prior exposure
Adeno-associated virus	All viral genes removed	Small genome limits size of foreign DNA
	Safe	Labor-intensive production
	Transduction of nondividing cells	Status of genome not fully elucidated
	Stable expression	
Nonviral		
Liposomes	Absence of viral components	Inefficient gene transfer into the nucleus
	Lack of previous immune recognition	Lack of persistence of DNA
		Lack of tissue targeting

Because of the complexity of the viral genome and toxicity associated with production of some of the viral proteins at sufficient levels to make the virus, removal of these genes has been more difficult than for retroviruses. How well these future-generation adenoviral vectors will function *in vivo* is currently being determined. Even if the genes are removed, there will be some level of immunity related to transgene production and the vector capsid itself. Thus, newer immunomodulatory agents that are more selective and are only administered for a brief period may be useful in blocking these immune responses, as demonstrated in several animal models (9).

Recombinant adeno-associated virus (AAV) vectors contain small, single-stranded DNA genomes and have recently been shown to transduce brain (10), skeletal muscle (11, 12), and liver (13) by injection into quiescent tissue or vasculature, feeding the tissue in animals. In fact, rAAV has been used to achieve therapeutic (14) or, in some cases, curative (13) concentrations of clotting factor IX in mice without toxicity for at least 9 months by *in vivo* delivery. Unfortunately, these vectors have a disadvantage in that there is a limit in the amount of DNA that can be packaged. Thus, larger cDNAs, genes, or complex regulatory cis elements cannot be used with this vector.

Regulation of gene expression may be important for treating some diseases, and recently, several different approaches to regulate gene expression have been used in animal models of gene expression. These approaches include the addition or subtraction of small molecules that interact with cis DNA elements and turn genes on or off. Moreover, tissue-specific regulation can be achieved by using cell-type-specific promoters or by designing vectors that specifically target an organ. Altering the tropism of the vector by constructing new ligands for receptor-specific targeting will certainly be important for future gene therapy applications.

There are a number of additional viral vectors based on Epstein–Barr virus, herpes, simian virus 40, papilloma, non-human lentiviruses, and hepatitis viruses that are currently being derived in the laboratory. Perhaps these chimeric or as yet undiscovered viruses will have properties that offer advantages to clinical gene therapy that are not yet realized.

Nonviral Vectors: Liposomes

Compared with viral vectors, cationic lipid-based delivery systems have several advantages. Unlike viral vectors, DNA/lipid complexes are easy to prepare and there is no limit to the size of genes that can be delivered. Because carrier systems lack proteins, they may evoke much less immunogenic responses. More importantly, the cationic lipid systems have much less risk of generating the infectious form or inducing tumorigenic mutations because genes delivered have low integration frequency and cannot replicate or recombine.

During the last few years, two classes of cationic lipids have been synthesized and show good transfection activity, which is mostly *in vitro* with established cell lines (15, 16). The first class has two alkyl chains in each cationic lipid molecule, and the other type uses cholesterol as the backbone. Both types of lipid contain either mono- or multiple-amino groups as the cationic function group to form complexes with DNA via electrostatic interactions. Each type of cationic lipid appears to have its preferred cell lines for an optimal transfection activity, even though both types of cationic lipids may show similar levels of transfection activity in a given cell type. With a few exceptions, the transfection activity of these cationic lipids is improved when a helper lipid, dioleoylphosphatidylethanolamine, is included as part of the liposome composition (17–19). In general, the transfection activity of these cationic liposomes *in vitro* is optimal with slight excess of cationic lipid in the DNA/lipid complexes (17–19).

Despite the early success of cationic lipid systems in transfecting cells mostly *in vitro* or *in vivo* by a local regional administration (20), no solid evidence has been presented in support of their gene transfer efficiency *in vivo* through systemic administration. This is largely due to some technical difficulty in preparing DNA/lipid complexes at concentrations where the injection volume into the animals is not too large and large aggregates will not form. However, such technical problems have been solved to a certain degree. A few recent reports have demonstrated that it is now possible to make a homogeneous solution with a higher concentration of DNA/lipid complexes by using either a much higher cationic-to-DNA ratio (21) than that used in *in vitro* transfection or by including into the lipids nonionic surfactants such as Tween 80 (22, 23) or other polyethylene-based amphiphiles (24). Using rodents, it has been shown that nanogram levels of gene product per milligram of proteins from the tissue extract can be achieved by a single tail vein injection of 25 μg of plasmid DNA containing the luciferase gene as a reporter (21, 25). Among the organs including lung, liver, spleen, heart, and kidney, the level of gene expression is the highest in the lung. Confocal microscopic study of the transfected lung using green fluorescence protein gene as the reporter indicates that the lung endothelial cells are the type of cells transfected (21). Time course shows that gene expression in different organs is transient, with the peak level between 8 and 20 hr and drops to less than 1% of the peak level by day 4. Results from Southern analysis suggest that such transient gene expression is likely due to the degradation of the transgene (21). However, the same level of gene expression can be regained by repeated injection after an interval of 2–3 weeks (21).

Although additional experiments are needed to show that gene expression *in vivo* by an intravenous administration will be useful for therapeutic purposes, these *in vivo* results reconfirm the potential of the lipid system as a carrier for gene therapy. As with

viral vectors, the next challenges are to achieve targeted gene delivery, to control the level of transgene expression, and to devise methods for long-term expression when needed.

Gene Therapy for Hematopoietic Derived Diseases

So far, most clinical trials on gene therapy focus on gene transfer into hematopoietic (blood cells) and cancer cells. In this report, we will illustrate the development of studies on gene transfer into hematopoietic stem cells. All hematopoietic cells arise from a single cell type designated as pluripotent hematopoietic stem cells (PHSCs). Therefore, successful stem cell gene therapy can be applied to a large variety of congenital and acquired blood cell diseases. In the last few years, the cell fraction that includes these PHSCs is being identified using molecules that are present on the cell surface; e.g., it has been found that PHSCs carry the CD34 antigen (CD34+; this is present in approximately 1% of the bone marrow cells) but are negative for other markers ("lin^{neg}, which is the case in approximately 1% of the CD34+ cells). By cell separation techniques, the CD34+lin^{neg} cells can be isolated and used for gene transfer studies. In these stem cell gene transfer studies, the cells are harvested from the patient, gene transfer is performed *ex vivo*, and the transduced cells are subsequently reinfused into the patients. The majority of studies use retroviral vectors as vehicles to mediate gene transfer. Initial trials aiming at gene transfer into hematopoietic cells focused on transfer of the gene encoding adenosine deaminase (ADA) (26–29). Deficiency of ADA results in a T cell defect, leading to a disease called severe combined immune deficiency. Patients with this genetic disease generally die in the first years of life due to overwhelming infections. At the moment these initial gene transfer trials started, gene transfer frequency into stem cells of larger animals such as dogs and monkeys was less than 3% (30–31). Despite this low frequency, clinical trials were initiated, because it was known from bone marrow transplantation experiments that "healthy cells" had a growth advantage over "diseased cells." Therefore, it was hoped that infusion of a low number of genetically repaired cells would result in the outgrowth of a functional immune system. So far, however, the frequency of transduced cells in blood is lower than the 3% reported in larger animal studies. A possible explanation may be that the "human hematopoietic stem cell" is more resistant to transduction than the animal's stem cells. It has also been suggested that, because the lack of pretreatment or conditioning used in animals may affect the outcome of the clinical studies, the transduced cells are only 0.001% of the endogenous stem cells of the patient, which is too low to result in detectable, stable engraftment. However, in similar experiments using animal models, the number of endogenous stem cells was reduced to virtually none by myeloablative therapy. Due to this myeloablation, the proportion of transduced cells following infusion was much higher and resulted in the long-lasting presence of transduced cells (32). Higher-efficiency gene transfer into human hematopoietic stem cells may occur during very long *ex vivo* cultivation of the stem cells over a bone marrow stroma cell layer.

Besides clinical studies in ADA-deficient patients, gene transfer into hematopoietic stem cells has been studied in so-called gene-marking studies (33). These studies are performed in patients who are treated for a malignancy with high-dose cytotoxic therapy, followed by reinfusion of their own stem cells that are harvested earlier in the course of the disease. In these patients, the majority of the graft is reinfused unmodified as a stem cell rescue, but a part of the graft is used for gene-transfer studies with marker genes. Also in these patients, the percentage of genetically marked blood cells after gene transfer is <1%. At present, it is generally believed that our limited knowledge in basic stem cell biology is the major bottleneck of stem cell gene transfer studies. For example, questions to be addressed include whether or not receptors for viral vectors are expressed and whether it is possible to trigger stem cells into cycle *ex vivo* without loss of pluripotency.

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Genetics

Ecdysone-inducible gene expression in mammalian cells and transgenic mice

(retinoid X receptor/tetracycline/promoter/cre recombinase)

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ABSTRACT **During metamorphosis of *Drosophila melanogaster*, a cascade of morphological changes is triggered by the steroid hormone 20-OH ecdysone via the ecdysone receptor, a member of the nuclear receptor superfamily. In this report, we have transferred insect hormone responsiveness to mammalian cells by the stable expression of a modified ecdysone receptor that regulates an optimized ecdysone responsive promoter. Inductions reaching 4 orders of magnitude have been achieved upon treatment with hormone. Transgenic mice expressing the modified ecdysone receptor can activate an integrated ecdysone responsive promoter upon administration of hormone. A comparison of tetracyline-based and ecdysone-based inducible systems reveals the ecdysone regulatory system exhibits lower basal activity and higher inducibility. Since ecdysone administration has no apparent effect on mammals, its use for regulating genes should be excellent for transient inducible expression of any gene in transgenic mice and for gene therapy.**

Precise control of gene expression is an invaluable tool in studying development and other physiological processes. Other applications for regulated gene expression include inducible gene targeting, overexpression of toxic and teratogenic genes, antisense RNA expression, and gene therapy (1). For cultured cells, glucocorticoids and other steroids are commonly used to induce expression of a desired gene. In the past several years, a tetracycline-regulated system has been devised in which gene activity is induced in the absence of the antibiotic and is repressed in its presence (2–5). Recently, the tetracycline-based system has been utilized in transgenic mice (4, 5). Disadvantages to this system include the continuous treatment of tetracycline to repress expression and the slow clearance of antibiotic from bone, which interferes with quick and precise inductions. While this system has been improved by the recent identification of a mutant tetracycline repressor, which acts conversely as an inducible activator, the pharmacokinetics of tetracycline may hinder its use during development when a precise and efficient on–off switch is essential (6).

As an alternative to tetracycline, we have explored the use of the insect molting hormone ecdysone as a potential inducer. A pulse of the steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing genomic effects such as chromosomal puffing within minutes of hormone addition (7–9). Mediating this response is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) (10, 11). Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or the synthetic analog muristerone A. Unlike tetracycline-based strategies, transferring ecdysone responsiveness to mammalian cells and transgenic mice takes advantage of a naturally evolved steroid-inducible system. Advantages for ecdysteroid use include the lipophilic nature of the compounds for efficient penetrance into all tissues including the brain, short half-lives which allow for precise and potent inductions, and favorable pharmacokinetics that prevent storage and expedite clearance. Because ecdysteroids are neither toxic, teratogenic, nor known to affect mammalian physiology, they would appear to be ideal candidates for use as inducers in cultured cells and transgenic mice (D.N., unpublished data). Here we report the development and properties of a regulatory system which reveals ecdysone to be an efficient and potent inducer of gene expression in cultured mammalian cells and transgenic mice.

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MATERIALS AND METHODS

Plasmids. CMX-EcR, CMX-USP, CMX-FXR, CMX-hRXRα, EcREx5-ΔMTV-Luc, CMX-GEcR, MMTV-Luc, and CMX-GR have been described (10, 12). CMX-VpEcR was constructed by ligation of an *Eco*RI fragment of psk-EcR and CMX-Vp16. CMX-VgEcR was generated by site-directed mutagenesis of CMX-VpEcR using the transformer mutagenesis kit (Clontech) and the mutagenic oligonucleotide 5′-TACAACGCCCTCACCTGTGGATCCTGCAAGG-TGTTTCTTTCGACGCAGC-3′. Mutagenesis of VpEcR to VgEcR altered the P box region of the DNA binding domain of EcR to resemble that of the glucocorticoid receptor (GR) (13). The following amino acids were altered: E282G, G283S, and G286V. EcREx4-ΔHSP-βgal was constructed by oligomerizing two annealed oligonucleotides containing the HSP-EcRE (10). EcREx4-Sp1x3-ΔHSP-βgal was made by ligating the following annealed oligonucleotides into the Asp718 site of EcREx4-ΔHSP-β-gal: 5′-GTACTCCCGGGGCGGGGC-TATGCGGGGCGGGGCTAATCGCTAGGGGCGGG-GCA-3′ and 5′-GTACTGCCCCGCCCCTAGCGATTAGC-CCCGCCCCGCATAGCCCCGCCCCGGGA-3′. ΔHSP is a minimal promoter derived from the *Drosophila* heat shock promoter with its enhancers deleted. To generate E/GREx4-ΔMTV-Luc, the oligonucleotides 5′-AGCTCGATGGACAAGTGCAT-TGTTCTTTGCTGAA-3′and5′-AGCTTTCAGCAAGAGAA-CAATGCACTTGTCCATCG-3′ were annealed, multimerized, and ligated into the *Hin*dIII site of ΔMTV-Luc. The resulting reporter contained four copies of the E/GRE. A *Bgl* II/(*Xho* I) fragment containing EcREx4-Sp1x3-ΔHSP-βgal was subcloned into *Bgl* II/(*Not* I)-digested pRC-CMV (Invitrogen), which contains a neomycin-resistance gene, resulting in construction of pRC-ESHβ. TETREx7-tk-luc, CMV-tTA-VP16, and CMV-rtTA-VP16 were obtained from H. Bujard (2–4).

Abbreviations: EcR, ecdysone receptor; GR, glucocorticoid receptor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-gal, β-galactosidase; RXR, retinoid X receptor; EcRE, ecdysone response element; FXR, farsenoid X receptor; GRE, glucocorticoid response element.

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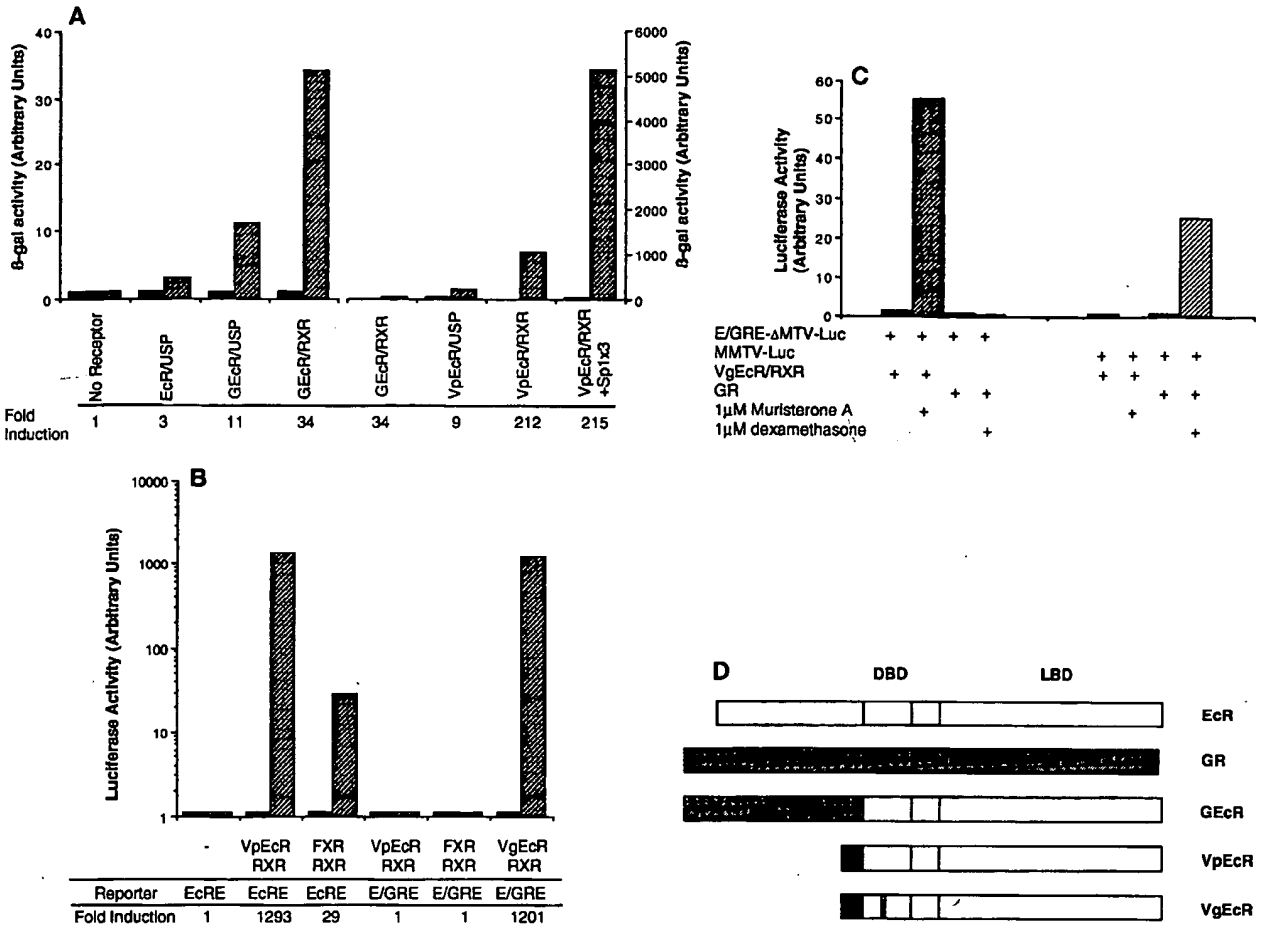


FIG. 1. (*A*) Optimization of ecdysone responsiveness using different modified EcR combinations. After transfection, cells were treated with either ethanol or 1 μM muristerone A. Numerical values on both sides of the figure are on the same scale, and the GEcR/RXR value was repeated for clarity. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A, respectively. (*B*) FXR and VpEcR activity on a EcRE and E/GRE responsive reporter. VpEcR, VgEcR, and the transfection without receptors were treated with 1 μM muristerone. FXR transfections were treated with 50 μM juvenile hormone III. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A/50 μM juvenile hormone III, respectively. (*C*) E/GRE and GRE are nonoverlapping response elements. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A/1 μM dexamethasone, respectively. (*D*) Schematic diagram of modified EcR derivatives. GEcR is a chimeric receptor containing the N-terminal transactivation domain of GR and the DNA and ligand binding domains of EcR. VpEcR is an N-terminal truncation of EcR fused to the activation domain of Vp16. VgEcR is identical to VpEcR except for the following point mutations in the P box of the DNA binding domain: E282G, G283S, and G286V. DBD, DNA binding domain; LBD, ligand binding domain.

Cell Culture and Transient Transfections. CV-1 cells were maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum (HyClone). Transient transfections were performed using Dotap transfection reagent (Boehringer

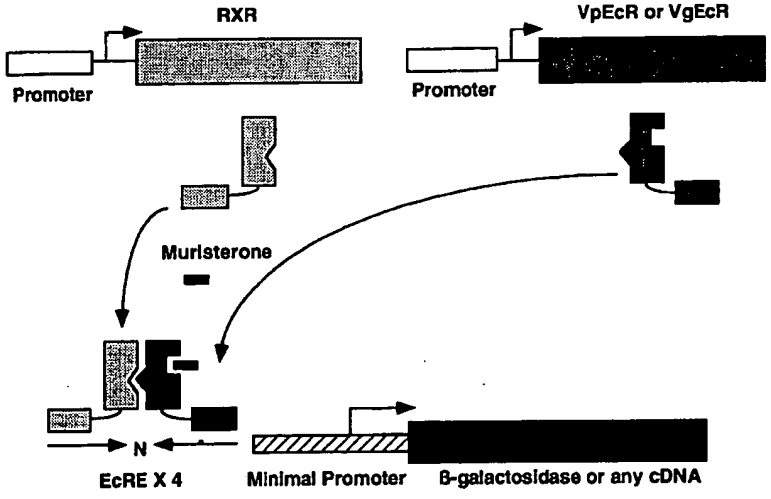
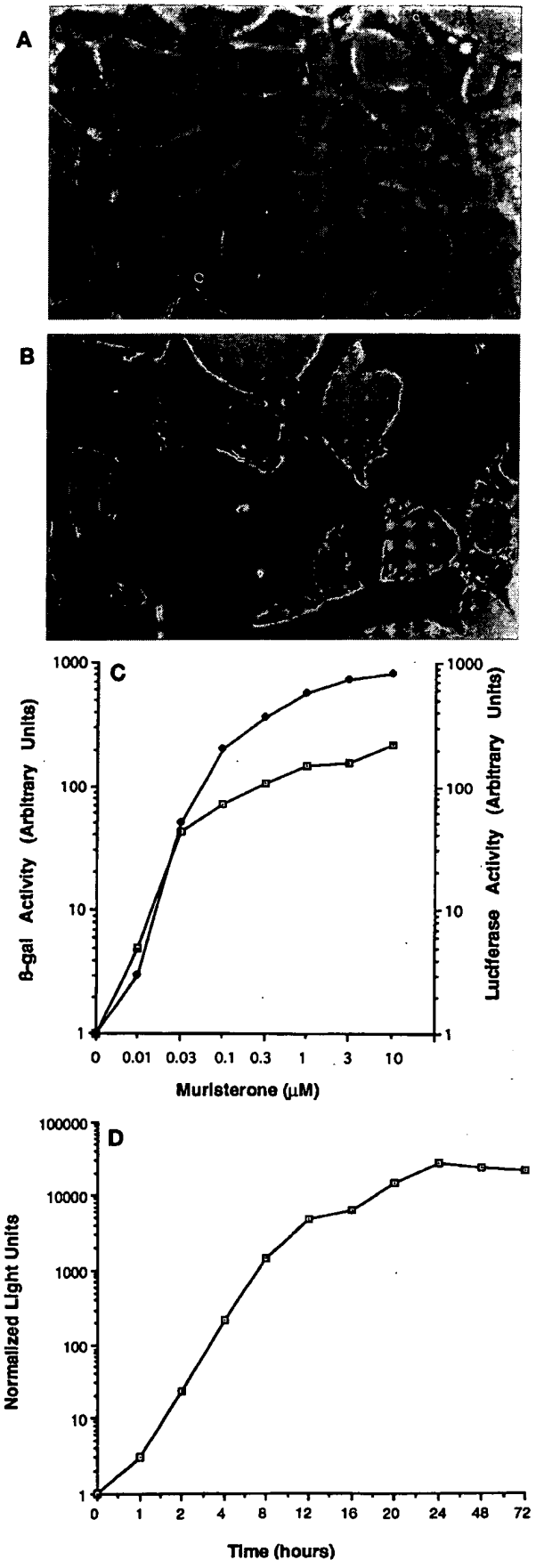


FIG. 2. Schematic diagram of ecdysone-inducible gene expression system. After expression of RXR and VpEcR, the two receptors can heterodimerize and transactivate the EcRE-containing promoter in the presence of hormone. The EcREs are placed upstream of a minimal promoter, which can drive the expression of any cDNA. For animals, two transgenic lines must be generated. The first line will express RXR and VpEcR. Tissue specificity will be conferred by selection of promoters that will then direct expression of the receptors. A second line will carry an ecdysone-responsive promoter controlling expression of a cDNA. Breeding of the two lines and treatment with muristerone will allow for temporal, dosage, and spatial specificity of cDNA expression.



Mannheim). Transfections using β-gal as the reporter were assayed either by Galactolight luminescent assay (Tropix) or by standard liquid *O*-nitrophenyl β-galactoside assay (ONPG) (Sigma). The values were normalized by cotransfection of CMX-luciferase. Transfections using luciferase as the reporter were assayed by standard techniques using luciferin and ATP. These values were normalized by cotransfection of CMX-β-gal. Hormone-treated cells were treated with ethanol, 50 μM juvenile hormone III (Sigma), 1 μM muristerone A (Zambon), or 1 μM dexamethasone (Sigma) unless otherwise noted. For comparison with the tetracycline repressor/activator, equimolar amounts of both reporter and activator plasmids were transfected. All transfections using doxycycline (Sigma) were performed in the dark as described (4).

Stable Cell Line Production. 293 cells were transfected with the linearized plasmids pRC-ESHβ allowed to recover 1 day prior to selection with G418 (1 mg/ml) (GIBCO). After 14 days of selection, 14 individual clones were isolated and grown separately in the presence of G418 (0.5 mg/ml). Of these, 10 exhibited muristerone responsiveness to differing degrees. Cell lysates were then assayed for β-gal and luciferase activities as already described. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining was performed on the stable cell lines. Cells were fixed briefly with 10% formaldehyde in PBS and then stained with X-Gal (Molecular Probes) for 2–6 hr.

Muristerone Treatment of Mice. All mice were treated with approved protocols of the Salk Institute for Biological Studies. For toxicity studies, adult mice were injected intraperitoneally with 20 mg of muristerone A suspended in sesame oil. The mice were then observed for ≈2 months. For teratogenic studies, pregnant mice were injected with 20 mg of muristerone A suspended in sesame oil and both the mother and pups were observed for 3 months. For muristerone bioavailability studies, adult mice were injected intraperitoneally with sesame oil with or without 10 mg of muristerone. After 12 hr, blood was drawn from the mice, and the serum was isolated by brief centrifugation of the whole blood. Serum from sesame oil injected mice was divided, and half was supplemented with muristerone to a final concentration of 10 μM. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRα, and EcREx5-ΔMTV-Luc.

Transgenic Mice. The following DNA constructs were prepared and subsequently injected into fertilized eggs: CD3-VpEcR, CD3-RXR, and ESHβ (14). CD3-VpEcR and CD3-RXR were mixed and coinjected while ESHβ was injected alone. Primary genotyping was performed by Southern blot analysis and transmission of transgenic mice was monitored by dot blot analysis. Expression of the receptors was determined by Northern blot analysis of RNA isolated from the thymus as well as other tissues for negative controls. Receptor-expressing mice were bred with reporter mice (containing ESHβ) to produce double-transgenic receptor/reporter mice. Double-transgenic lines were treated with muristerone A as described above and RNA was isolated 48 hr after hormone treatment. For Northern blot analysis, 15 μg of total RNA from various tissues including the thymus was run on a denaturing gel and

FIG. 3. (*A* and *B*) X-Gal staining of stable cell line N13. Of 14 G418-resistant clones, 10 demonstrated muristerone responsiveness. One of these cell lines, N13, was grown with (*A*) or without (*B*) the presence of 1 μM muristerone for 20 hr. The cells were then fixed and treated with X-Gal for 2 hr. (*C*) Dose-dependent activation of N13 cells with muristerone. N13 cells were grown with varying concentrations of muristerone for 36 hr and then assayed for β-gal activity (open squares) by standard *O*-nitrophenyl β-galactoside assay or for luciferase activity (solid circles). (*D*) Time course of luciferase activity of N13 cells treated with hormone. N13 cells were grown in separate wells in the presence of 1 μM muristerone, harvested at varying times, and assayed for luciferase activity.

blotted onto a nitrocellulose membrane. The blot was probed with a radiolabeled β-gal-specific probe and exposed on film for 2 days.

RESULTS

Optimization of EcR Derivatives. Previous studies have shown that mammalian cells cotransfected with EcR and USP produce a 3-fold induction upon treatment with 1 μM muristerone, an ecdysone analog (10, 11) (Fig. 1*A*). To maximize the sensitivity of an ecdysone-inducible system, modifications of EcR were made. The N-terminal transactivation domain of EcR was replaced by the corresponding domain of GR (Fig. 1*D*). This new hybrid receptor named GEcR boosted muristerone responsiveness from 3- to 11-fold in a transient transfection assay (Fig. 1*A*). Replacement of EcR's natural heterodimeric partner, USP, by its mammalian homologue, the retinoid X receptor (RXR), produced a more potent ligand-dependent heterodimer, producing a 34-fold induction (Fig. 1*A*). A most potent heterodimer, however, was a combination of RXR and VpEcR, an N-terminal truncation of EcR attached to the VP16 activation domain resulting in a 212-fold induction (Fig. 1 *A* and *D*). Different from most nuclear receptor/VP16 fusion proteins, which exhibit high constitutive activity, VpEcR generates ligand-dependent superinduction while maintaining a very low basal activity (15, 16). In addition, the reporter vector was also modified by inserting consensus binding sites for the ubiquitous transcription factor Sp1 between the minimal promoter and the ecdysone response elements (EcREs). Sp1 has been shown to boost transcriptional activity of many promoters (17–19). The addition of Sp1 sites to the ecdysone responsive promoter increases the absolute activity 5-fold (Fig. 1*A*).

Construction of a Novel Ecdysone-Specific Response Element. Although no mammalian transcription factors have been shown to have a natural enhancer element like the EcRE, which is composed of two inverted half-sites of the sequence AGGTCA spaced by 1 nucleotide, it is difficult to preclude such a possibility. The recently cloned farsenoid X receptor (FXR) can very weakly activate certain synthetic promoters containing an EcRE in response to extremely high concentrations of farsenoids (14). For FXR-containing cells and transgenic mice, it would be undesirable if endogenous receptors can activate an EcRE-containing promoter. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR, which binds as a homodimer to an inverted repeat of the sequence AGAACA spaced by 3 nucleotides. This altered binding specificity was achieved by mutating 3 amino acid residues of VpEcR in the P box of the DNA binding domain, a region previously shown to be essential for DNA sequence recognition (13). This hybrid receptor is named VgEcR and is responsive to a hybrid responsive element called the E/GRE, which contains two different half-sites, AGGTCA and AGAACA, spaced by 1 nucleotide (Fig. 1*B*). This response element is a hybrid between the glucocorticoid response element (GRE) and that of type II nuclear receptors like RXR, EcR, retinoic acid receptor, thyroid hormone receptor, etc. Although FXR can activate a promoter containing the wild type EcRE, it cannot activate one containing the E/GRE (Fig. 1*B*; note logarithmic scale). The E/GRE reporter is not activated by GR nor does VgEcR activate a dexamethasone responsive promoter (Fig. 1*C*).

Ecdysone Responsiveness in Stable Cell Lines. Stable cell lines were generated containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and an ecdysone-inducible reporter (Fig. 2). X-Gal staining of one stable cell clone, N13, was performed. After 24 hr of treatment with 1 μM muristerone, 100% of the cells turned dark blue with 3 hr of staining (Fig. 3 *A* and *B*). Dose–response curves of stably integrated β-gal and luciferase reporters in N13 cells revealed

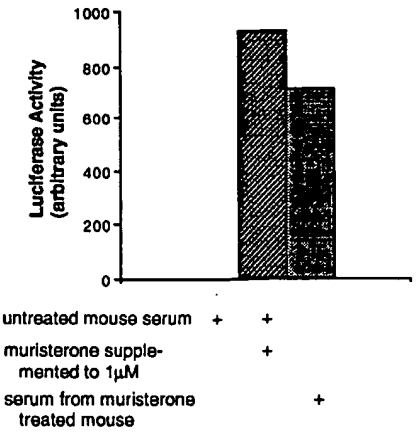


FIG. 4. Muristerone activity in mice. Adult mice were injected intraperitoneally with sesame oil with or without 10 mg of muristerone. After 12 hr, blood was drawn from the mice, and the serum was isolated. Serum from sesame oil-injected mice was divided, and half was supplemented with muristerone to a final concentration of 10 μM. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRα, and EcREx5-ΔMTV-Luc.

that inducibility approaching 3 orders of magnitude can be achieved at a final concentration of 10 μM muristerone (Fig. 3*C*). One-hundred-fold induction was achieved by muristerone concentrations as low as 100 nM (Fig. 3*C*). Finally, the kinetics of muristerone-mediated induction was measured. Inductions of 100-fold in 3 hr, 1000-fold in 8 hr, and maximal effects of 20,000-fold after 20 hr of treatment were seen (Fig. 3*D*). Similar results were seen in stable lines containing CMX-VgEcR and the E/GRE reporters.

Bioavailability and Activity of Muristerone A. To use muristerone as a potential hormone in mice, its bioavailability was examined. Adult mice were injected intraperitoneally with muristerone and were subsequently sacrificed for serum collection. Serum taken 12 hr after injection was used in a transfection assay to test for muristerone activity. Serum from muristerone-

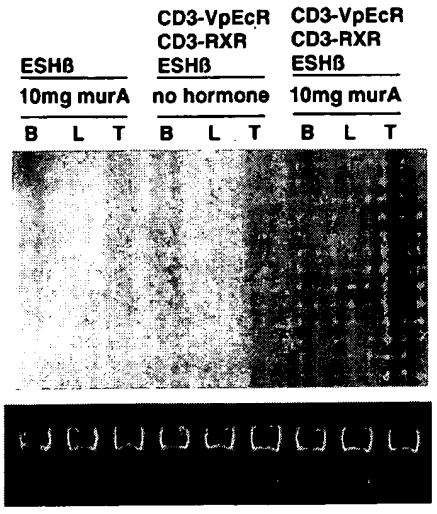


FIG. 5. Muristerone-dependent gene expression in transgenic mice. (*Upper*) Northern blot analyses were performed using 10 μg of total RNA isolated 48 hr after muristerone or sesame oil treatment. The probe used was specific to activity of the ecdysone-inducible promoter. Genotypes of the animals are described as shown. Autoradiograph was exposed for 36 hr. (*Lower*) Ethidium bromide staining of RNA prior to blotting. B, brain; L, liver; T, thymus.

treated mice yielded a luciferase activity comparable to that seen from untreated mouse serum supplemented with 1 μM muristerone (Fig. 4). Thus, not only should single-site injected material be widely circulated, but also there appears to be little or no blunting of activity due to association with serum proteins.

Muristerone-Dependent Gene Expression in Transgenic Mice. Transgenic mice were generated harboring an ecdysone-inducible reporter, ESHβ, or a T-cell-specific expression construct of VpEcR and RXR. The former will be referred to as reporter mice, the latter will be referred to as receptor mice, and double-transgenic mice will be referred to as receptor/reporter mice. Receptor mice were analyzed for VpEcR and RXR expression by Northern blot analysis of RNA collected from these mice (data not shown). These receptor mice were healthy, fertile, and by gross appearance normal. In addition, the transgene was transferred to the offspring as expected by Mendelian genetics (data not shown). This suggests that overexpression of VpEcR and RXR in T cells is not toxic. Receptor mice were then bred with reporter mice to produce double-transgenic receptor/reporter mice. After treatment with 10 mg of muristerone, RNA was isolated from various tissues including the thymus to test for specific induction of an ecdysone-inducible promoter. Muristerone treatment caused a significant induction from an ecdysone-inducible promoter, while low basal activity is observed in its absence (Fig. 5).

Comparison of Tetracycline-Based vs. Ecdysone-Inducible Systems. Transient transfection assays were performed to compare the relative activity and inducibility of the tetracycline repressor (tTA), tetracycline activator (rtTA), and modified ecdysone receptor (VgEcR/RXR) (2–4). The tetracycline repressor (tTA) activates a reporter containing tetracycline response elements (TETREs) in the absence of doxycycline, yielding a 59-fold activation (Fig. 6). This activation was completely repressed when doxycycline (0.01 μg/ml) was supplemented to the medium. In contrast, rtTA significantly activated the same reporter in the absence of doxycycline. Upon treatment with doxycycline (1 μg/ml), there was a 2.5-fold increase in activation above basal activity. This modest inducibility was due to high basal activity of rtTA, which approached maximal activity by tTA. Transfections with the ecdysone-inducible promoter (E/GRE) exhibited basal activity that was 20-fold lower than tTA and 500-fold lower than rtTA. Treatment of 1 μM muristerone A boosted reporter activity almost 1000-fold.

DISCUSSION

Tightly regulated gene expression by an exogenous inducer has numerous uses. For example, inducible expression of the cre recombinase in transgenic mice would allow for temporally specific inducible gene targeting of the adult or the developing embryo (20). Inducible expression of toxins such as the diphtheroid toxin would allow for inducible tissue-specific ablation (21).

We report here the use of the insect steroid hormone ecdysone as a potent inducer of gene activation in mammalian cells and transgenic mice. The optimized promoters containing a novel response element and the Vp16/EcR fusion receptor with an altered DNA binding specificity have yielded an extremely powerful and specific inducible system. The system's low basal activity is ideal for expression of transcription factors and toxic genes. This is likely due to the consequence that DNA binding and activation of EcR requires hormone (10). The excellent dose–response and induction rate characteristics of the ecdysone-inducible system will allow for precise control of both the amount and time period for which a desired gene is induced.

Because steroid hormones have evolved to efficiently penetrate virtually all tissues, EcR agonists like muristerone A will be useful in the study of embryonic development of any organ, including the brain. We have shown that muristerone maintains its activity when injected into mice and that it is neither toxic, teratogenic, nor inactivated by serum binding proteins. In addition to the inert qualities of muristerone, overexpression of VpEcR and RXR appears not to be toxic, at least in T cells. In transgenic mice containing an ecdysone-inducible promoter and expression of VpEcR and RXR, muristerone treatment can activate gene expression. Thus, with tissue-specific expression of VpEcR and RXR and timely hormone treatment, inducible gene expression can be achieved with spatial and temporal specificity.

In contrast to tetracycline-based systems, steroids like ecdysone offer pharmacokinetics that allow for both fast distribution and clearance of the inducer. In a direct comparison, VgEcR/RXR demonstrated both lower basal activity and higher inducibility than tTA or rtTA. Finally, in conjunction with the tetracycline-based systems, the ecdysone-inducible

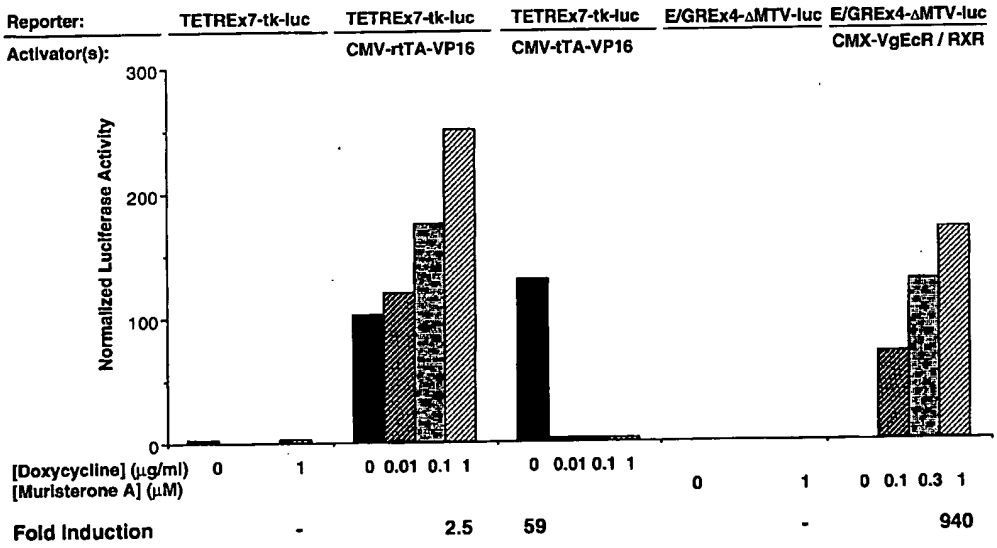


FIG. 6. Comparison of tetracycline-based and ecdysone-based inducible systems. In a transient transfection assay, CV-1 cells were transfected with equimolar amounts of TETREx7-tk-luc, E/GREx4-ΔMTV-luc, CMV-tTA-VP16, CMV-rtTA-VP16, CMX-VgEcR, and CMX-RXR, respectively. Ten times more reporter plasmid was transfected than activator(s) plasmid(s). After 24-hr hormone treatment, cells were lysed and assayed for luciferase and β-gal activities. All values have been normalized by cotransfection with CMX-β-gal.

system will allow for induction of two different genes in a temporal, spatial, and dosage-specific manner.

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Agenda for Meeting October 28, 1999 with Applicants' Representative Stephen Reiter 3:30PM

RE: Application No. 08/973,530: Attorney Docket No. SALK1520-1 and
Application No. 09/042,488: Attorney Docket No. SALK1520-2

1. Discuss Office Action mailed 7/2/99 in Application No. 09/042,488

   A. Statutory type double patenting rejections.
   - Claims 25, 26, 29, 31 and 33 provisionally rejected as claiming the same invention as that of claims 1, 5-13, 16 and 17 of co-pending Application No. 08/974,530 (SALK1520-1);
     Discuss proposed disposition of patent applications related to '488.
   - Claims 1-8, 11-14, 19-20, 22-24 are provisionally rejected as claiming the same invention as that of claims 1-2, 4, 8-10, 15-26 of copending Application No. 08/628,830 (SALK1520);
     Discuss proposed disposition of patent applications related to '488.

   B. Non-statutory type double patenting rejection of claims 15, 17, 27, 28, 30, 32 and 34 over claims 1-18 of copending application 08/974,530 in view of *Mikitani;*
   - Clarify how rejected claims distinguish over patent application; and
   - Consider applicability of *Mikitani* to these claims.

   C. Rejections of claims 1-34 under 35 U.S.C. § 112, first paragraph as allegedly not enabling therapeutic method because method "lies in the realm of gene therapy".
   - Discuss support desired by Examiner; and
   - Discuss enablement provided by the specification.

   D. Rejection of claims 25-29, 31-34 under 35 U.S.C. § 102 as allegedly being anticipated by Meybeck et al (U.S. Patent No 5,198,225);
   - Discuss how claims distinguish over reference.

   E. Rejection of claims 25-34 under 35 U.S.C. § 103 as allegedly being obvious over *Mikitani;*
   - Discuss how claims distinguish over reference.

2. Discuss status of Application No. 08/974,530 in view of Applicants' response to Office Action, filed 8/27/99 and amendments therein; discuss role of this application in view of pending related applications.